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(54) Title: GLYCINE TRANSPORTER-TRANSFECTED CELLS AND USES THEREOF

(57) Abstract

The present invention relates to materials and methods for the identification of agents that regulate glycine transport in or out of cells, particularly in or out of neuronal and neuronal-associated cells. Such materials include non-mammalian cells having transfected therein a glycine transporter. The methods relate to the manipulation of such cells such that agents are identified that cause intake or outflow of glycine with respect to a given glycine transporter.

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GLYCINE TRANSPORTER-TRANSFECTED CELLS AND USES THEREOF

The present invention relates to the field of drug discovery, particularly with respect to drugs that have an effect on glycine-mediated neurotransmission in the nervous system.

Synaptic transmission is a complex form of intercellular communication that involves a considerable array of specialized structures in both the pre- and post-synaptic neuron. High-affinity neurotransmitter transporters are one such component, located on the pre-synaptic terminal and surrounding glial cells (Kanner and Schuldiner, CRC Critical Reviews in Biochemistry, 22, 1032 (1987)). Transporters sequester neurotransmitter from the synapse, thereby regulating the concentration of neurotransmitter in the synapse, as well as its duration therein, which together influence the magnitude of synaptic transmission. Further, by preventing the spread of transmitter to neighboring synapses, transporters maintain the fidelity of synaptic transmission. Last, by sequestering released transmitter into the presynaptic terminal, transporters allow for transmitter reutilization.

Neurotransmitter transport is dependent on extracellular sodium and the voltage difference across the membrane; under conditions of intense neuronal firing, as, for example, during a seizure, transporters can function in reverse, releasing neurotransmitter in a calcium-independent non-exocytotic manner (Attwell et al., Neuron, 11, 401-407 (1993)). Pharmacologic modulation of neurotransmitter transporters thus provides a means for modifying synaptic activity, which provides useful therapy for the treatment of neurological and psychiatric disturbances.

The amino acid glycine is a major neurotransmitter in the mammalian nervous system, functioning at both inhibitory and

excitatory synapses. By nervous system, both the central and peripheral portions of the nervous system are intended. These distinct functions of glycine are mediated by two different types of receptor, each of which is associated with a different class of glycine transporter. The inhibitory actions of glycine are mediated by glycine receptors that are sensitive to the convulsant alkaloid, strychnine, and are thus referred to as "strychnine-sensitive." Such receptors contain an intrinsic chloride channel that is opened upon binding of glycine to the receptor; by increasing chloride conductance, the threshold for firing of an action potential is increased. Strychnine-sensitive glycine receptors are found predominantly in the spinal cord and brainstem, and pharmacological agents that enhance the activation of such receptors will thus increase inhibitory neurotransmission in these regions.

Glycine functions in excitatory transmission by modulating the actions of glutamate, the major excitatory neurotransmitter in the central nervous system. See Johnson and Ascher, Nature, 325, 529-531 (1987); Fletcher et al., Glycine Transmission, (Otterson and Storm-Mathisen, eds., 1990), pp. 193-219. Specifically, glycine is an obligatory co-agonist at the class of glutamate receptor termed N-methyl-D-aspartate (NMDA) receptor. Activation of NMDA receptors increases sodium and calcium conductance, which depolarizes the neuron, thereby increasing the likelihood that it will fire an action potential. NMDA receptors are widely distributed throughout the brain, with a particularly high density in the cerebral cortex and hippocampal formation.

Molecular cloning has revealed the existence in mammalian brains of two classes of glycine transporters, termed GlyT-1 and GlyT-2. GlyT-1 is found predominantly in the forebrain, and its distribution corresponds to that of glutamatergic pathways and NMDA receptors

(Smith, et al., Neuron, 8, 927-935 (1992)). Molecular cloning has further revealed the existence of at least three variants of GlyT-1, termed GlyT-1a, GlyT-1b and GlyT-1c (Kim et al., Molecular Pharmacology, 45, 608-617 (1994)), each of which displays a unique distribution in the brain and peripheral tissues. These variants arise by differential splicing and exon usage, and differ in their N-terminal regions. GlyT-2, in contrast, is found predominantly in the brain stem and spinal cord, and its distribution corresponds closely to that of strychnine-sensitive glycine receptors (Liu et al., J. Biological Chemistry, 268, 22802-22808 (1993); Jursky and Nelson, J. Neurochemistry, 64, 1026-1033 (1995)). These data are consistent with the view that, by regulating the synaptic levels of glycine, GlyT-1 and GlyT-2 selectively influence the activity of NMDA receptors and strychnine-sensitive glycine receptors, respectively.

Sequence comparisons of GlyT-1 and GlyT-2 have revealed that these glycine transporters are members of a broader family of sodium-dependent neurotransmitter transporters, including, for example, transporters specific for γ-amino-n-butyric acid (GABA) and others. Uhl, Trends in Neuroscience, 15, 265-268 (1992); Clark and Amara, BioEssays, 15, 323-332 (1993). Overall, each of these transporters includes 12 putative transmembrane domains that predominantly contain hydrophobic amino acids. Comparing rat GlyT-1a or rat GlyT-1b to rat GlyT-2, using the Lipman-Pearson FASTA algorithm, reveals a 51% amino acid sequence identity and a 55% nucleic acid sequence identity. Comparison of the sequence of human GlyT-1a, human GlyT-1b, or human GlyT-1c with rat GlyT-2 reveals in each case a 51% amino acid sequence identity and a 53-55% nucleic acid s quence identity. However, there are segments of human GlyT-1c 16 amino acids in length whose amino acid sequences are

100% identical to those of rat GlyT-2; the corresponding nucleic acid sequence of this region, which is 48 nucleotides in length, is 78-85% identical between the two transporters. A yet longer stretch of approximately 260 amino acids displays 53% amino acid sequence identity between human GlyT-1c and rat GlyT-2; the corresponding nucleotide sequence for this region, 780 nucleotides in length, displays about 66% sequence identity between the two transporters.

Compounds that inhibit or activate glycine transporters would be expected to alter receptor function, and provide therapeutic benefits in a variety of disease states. For example, inhibition of GlyT-2 can be used to diminish the activity of neurons having strychnine-sensitive glycine receptors via increasing synaptic levels of glycine, thus diminishing the transmission of pain-related (*i.e.*, nociceptive) information in the spinal cord, which has been shown to be mediated by these receptors. Yaksh, Pain, 111-123 (1989). Additionally, enhancing inhibitory glycinergic transmission through strychnine-sensitive glycine receptors in the spinal cord can be used to decrease muscle hyperactivity, which is useful in treating diseases or conditions associated with increased muscle contraction, such as spasticity, myoclonus, and epilepsy (Truong et al., Movement Disorders, 3, 77-87 (1988); Becker, FASEB J., 4, 2767-2774 (1990)). Spasticity that can be treated via modulation of glycine receptors is associated with epilepsy, stroke, head trauma, multiple sclerosis, spinal cord injury, dystonia, and other conditions of illness and injury of the nervous system.

NMDA receptors are critically involved in memory and learning (Rison and Stanton, Neurosci. Biobehav. Rev., 19, 533-552 (1995); Danysz t al., Behavioral Pharmacol., 6, 455-474 (1995)); and, furthermore, decreased function of NMDA-mediated neurotransmission

appears to underlie, or contribute to, the symptoms of schizophrenia (Olney and Farber, Archives General Psychiatry, 52, 998-1007 (1996)). Thus, agents that inhibit GlyT-1 and thereby increase glycine activation of NMDA receptors can be used as novel antipsychotics and anti-dementia agents, and to treat other diseases in which cognitive processes are impaired, such as attention deficit disorders and organic brain syndromes. Conversely, over-activation of NMDA receptors has been implicated in a number of disease states, in particular the neuronal death associated with stroke and possibly neurodegenerative diseases, such as Alzheimer's disease, multi-infarct dementia, AIDS dementia, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis or other conditions in which neuronal cell death occurs, such as stroke and head trauma. Coyle & Puttfarcken, Science, 262, 689-695 (1993); Lipton and Rosenberg, New Engl. J. of Medicine, 330, 613-622 (1993); Choi, Neuron, 1, 623-634 (1988). Thus, pharmacological agents that increase the activity of GlyT-1 will result in decreased glycine-activation of NMDA receptors, which activity can be used to treat these, and related, disease states. Similarly, drugs that directly block the glycine site on the NMDA receptors can be used to treat these and related disease states.

Methods and materials are needed to identify the aforementioned pharmacological agents. In particular, a drug screening method or methods relating to the identification of pharmacological agents that regulate glycine transport or interact with glycine receptors are needed.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to materials and methods for the identification of agents that regulate glycine transport in or out of

cells, or that interact with glycine receptors. Such materials include cells having transfected therein a glycine transporter. The methods relate to the manipulation of such cells such that agents are identified that inhibit or stimulate intake or outflow of glycine with respect to a given glycine transporter.

In a preferred embodiment, the present invention relates to a non-mammalian cell comprising an exogenous nucleic acid encoding a glycine transporter. Such an embodiment allows for the specific demonstration of the activity of a mammalian transporter in a genetically different background. A non-mammalian cell of the present invention is selected from the group consisting of avian, fungal, insect, and reptilian; most preferably the cell is avian. Preferably, the exogenous nucleic acid of the present invention is mammalian; more preferably the exogenous nucleic acid is human or rat. As noted, the inventive non-mammalian cell includes the glycine transporter, which is glycine transporter-1 (GlyT-1) or glycine transporter-2 (GlyT-2), wherein GlyT-1 is preferably GlyT-1a, GlyT-1b, or GlyT-1c. Preferably, the glycine transporter is GlyT-1, wherein the exogenous nucleic acid is preferably selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3. In another embodiment, the glycine transporter is GlyT-2, wherein the exogenous nucleic acid is preferably SEQ ID NO:4. The non-mammalian cell of the present invention preferably is a quail fibroblast, and most preferably is a QT-6 cell.

Another preferred embodiment of the present invention relates to a method for the analysis or screening of an agent for treatment of pain, muscle hyperactivity, neuronal cell death, schizophrenia, memory or cognitive disorders, or other disorders or conditions associated with a nervous system disorder or condition, comprising culturing separately first and second non-mammalian cells,

wherein the first and second non-mammalian cells are of the same strain and comprise an exogenous nucleic acid encoding a glycine transporter, contacting the first non-mammalian cell with the agent, and screening for the enhancement or inhibition of glycine transport into the first non-mammalian cell as compared to glycine transport into the second non-mammalian cell that was not contacted with the compound. The nervous system disorder or condition noted hereinabove is selected from the group consisting of spasticity, muscle spasm, myoclonus, epilepsy, stroke, head trauma, multiple sclerosis, spinal cord injury, dystonia, Alzheimer's disease, multi-infarct dementia, AIDS dementia, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Preferably, the glycine transporter used in the context of this method is GlyT-1 or GlyT-2, wherein GlyT-1 is preferably GlyT-1a, GlyT-1b, or GlyT-1c. A further preferred embodiment of the present invention includes first and second non-mammalian cells comprising exogenous nucleic acid that encodes GlyT-1, such as exogenous nucleic acid that comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3. Alternatively, the first and second non-mammalian cells of the present invention includes exogenous nucleic acid that encodes GlyT-2, such as exogenous nucleic acid that comprises SEQ ID NO:4. In a preferred embodiment, the non-mammalian cell of the present invention is a QT-6 cell. In yet a further preferred embodiment, the drug discovered by the inventive method is an enhancer or inhibitor of GlyT-1 or GlyT-2 or both GlyT-1 and GlyT-2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are bar graphs that depict the results of heterologous expr ssion of glycine transporters in QT-6 cells.

Figures 2A and 2B are graphs that respectively depict the attenuation of MK-801 binding by a glycine site antagonist (Figure 2A) and the potentiation of MK-801 binding by a glycine site agonist (Figure 2B).

Figure 3 is a bar graph that depicts NMDA receptor-mediated calcium uptake in primary neuronal cell cultures, and its blockade by the glycine site antagonist L-689,560.

DETAILED DESCRIPTION

The present invention is directed to materials and methods for the identification of agents that regulate glycine transport in or out of cells or that interact with glycine receptors. In particular, such glycine transport is mediated or caused by action of the glycine transporter type 1 (GlyT-1) or glycine transporter type 2 (GlyT-2). GlyT-1 has been found to express as at least three different isoforms that differ in their 5' ends; accordingly, "GlyT1" as used herein refers to, but is not limited to GlyT-1a [SEQ ID NO:1], GlyT-1b [SEQ ID NO:2], GlyT-1c [SEQ ID NO:3], and other such variants that are similarly related in sequence and transcriptional origin. For example, GlyT-1a is transcribed from a different promoter than is GlyT-1b and GlyT-1c; however, all three isoforms differ by differential splicing and exon usage. Adams et al., J. Neurosci., 15, 2524-2532 (1995); Kim et al., Molec. Pharmacol., 45, 608-617 (1994). Thus, other such variants contemplated as preferred glycine transporters used in the context of the present invention are further isoforms that differ by differential splicing and exon usage.

The glycine transporter genes and their respective gene products are responsible for the reuptake of glycine from the synaptic cleft into presynaptic nerve endings or glial cells, thus terminating the action of glycine. Neurological disorders or conditions associated with

improperly controlled glycine receptor activity, or which could be treated with therapeutic agents that modulate glycine receptor activity, include spasticity (Becker, FASEB Journal, 4, 2767-2774 (1990)) and pain realization (Yaksh, Pain, 37, 111-123 (1989)). Additionally, glycine interacts at N-methyl-D-aspartate (NMDA) receptors, which have been implicated in learning and memory disorders and certain clinical conditions such as epilepsy, Alzheimer's and other cognition-related diseases, and schizophrenia. See Rison and Stanton, Neurosci. Biobehav. Rev., 19, 533-552 (1995); Danysz et al., Behavioral Pharmacol., 6, 455-474 (1995).

Compounds that inhibit GlyT-1 mediated glycine transport will increase glycine concentrations at NMDA receptors, which receptors are located in the forebrain, among other locations. This concentration increase elevates the activity of NMDA receptors, thereby alleviating schizophrenia and enhancing cognitive function. Alternatively, compounds that interact directly with the glycine receptor component of the NMDA receptor can have the same or similar effects as increasing or decreasing the availability of extracellular glycine caused by inhibiting or enhancing GlyT-1 activity, respectively. See, for example, Pitkänen et al., Eur. J. Pharmacol., 253, 125-129 (1994); Thiels et al., Neuroscience, 46, 501-509 (1992); and Kretschmer and Schmidt, J. Neurosci., 16, 1561-1569 (1996). Compounds that inhibit GlyT-2 mediated glycine transport will increase glycine concentrations at receptors located primarily in the brain stem and spinal cord, where glycine acts as an inhibitor of synaptic transmission. These compounds are effective against epilepsy, pain and spasticity, and other such conditions. See, for example, Becker, *supra*, and Yaksh, *supra*.

Accordingly, the identification of agents that enhance or inhibit the glycine transporter, or inhibit or activate the glycine receptor portion

of the NMDA receptor, is important for the development of drugs useful in the treatment of such neurological conditions and disorders. The present invention provides materials and methods that are suitable for such screening. In particular, GlyT1, which is preferably GlyT-1a, -1b, or -1c, and GlyT-2 DNA sequences, when placed into a suitable expression vector and a suitable host is transformed therewith, the GlyT1, preferably GlyT-1a, -1b, or -1c, and GlyT-2, respectively, glycine transporter polypeptides are synthesized and form the respective glycine transporter. Such transformed cells may form stable lines that constitutively or inductively express the GlyT DNA, thus expressing glycine transporters. Alternatively, other such cells may exhibit transient expression of the GlyT DNA and protein. Either of such transfected cells, together or separately, are useful for screening assays to determine whether a candidate agent has characteristics of enhancing or inhibiting glycine transport, as disclosed herein with respect to the present invention. Additionally, suitable primary neuronal cell cultures that have NMDA receptors and glycine transporters are also used in the context of the present invention to test compounds for the ability to activate or inhibit either the glycine transporter, the glycine receptor portion of the NMDA receptor, or both. Such tests also have the form of binding assays using membranes from any suitable source that includes NMDA receptors, such as brain tissue.

Suitable expression vectors include pRc/CMV (Invitrogen), pRc/RSV (Invitrogen), pcDNA3 (Invitrogen), Zap Express Vector (Stratagene Cloning Systems, LaJolla, CA; hereinafter "Stratagene"), pBk/CMV or pBk-RSV vectors (Stratagene), Bluescript II SK +/- Phagemid Vectors (Stratagene), LacSwitch (Stratagene), pMAM and pMAM neo (Clontech), among others. A suitable expression vector is capable of fostering xpression of the included GlyT DNA in a suitable

host cell, preferably a non-mammalian host cell, which can be eukaryotic, fungal, or prokaryotic. Such preferred host cells include, but are not limited to, avian, fungal, insect, and reptilian cells. Preferred host cells are avian, fungal, and insect cells. Most preferred host cells are avian cells. Preferred avian cells include those of quails, chickens, and turkeys; more preferred, of quails. Most preferred of such cells are quail fibroblast, such as, in particular, QT-6.

The GlyT DNA that is inserted into one of the aforementioned expression vectors is any suitable DNA that encodes a glycine transporter. Preferably, the GlyT DNA is obtained from a suitable animal, including but not limited to birds and mammals, for example. Preferred mammals include humans, mice, rats, cows, pigs, among others; more preferably, the GlyT DNA is obtained from a human or a rat; most preferably, the GlyT DNA is obtained from a human. In one embodiment, the GlyT DNA is preferably comprised of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, with respect to GlyT-1, and SEQ ID NO:4, with respect to GlyT-2. Any other suitable DNA that encodes glycine transporter type 1 activity is an equivalent substitution for SEQ ID Nos:1-3. Similarly, any other suitable DNA that encodes glycine transporter type 2 activity is an equivalent substitution for SEQ ID NO:4.

In another embodiment, the GlyT DNA used in the context of the present invention encodes a protein that has at least about 45% amino acid sequence identity with at least one of the proteins encoded by SEQ ID NOs:1-4, more preferably at least about 60% amino acid sequence identity, still more preferably at least about 75% amino acid sequence identity, yet still more preferably at least about 85% amino acid sequence identity. Sequence identity measurements as contemplated herein score conservative amino acid substitutions as

identical, wherein conservative substitutions are those that cause exchanges of amino acids in the encoded protein, which amino acids have highly similar physicochemical characteristics or have been known empirically to substitute in homologous proteins. At the nucleic acid level, exchanges of nucleotides can occur that are neutral in their effect on the encoded protein sequence, in consequence of the redundancy of the genetic code, which could account for greater sequence variation at the nucleic acid level than at the amino acid level.

Such exchangeable amino acids are categorized within one of the following groups, wherein the amino acids are recited by their respective three-letter codes that are well known in the art:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly;
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;
3. Polar, positively charged residues: His, Arg and Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and
5. Aromatic residues: Phe, Tyr and Trp.

A preferred listing of conservative substitutions, based on empirical evidence from studies on homologous protein sequences, is the following:

Original Residue	**Substitution**
Ala	Gly, Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn

Original Residue	Substitution
Glu	Asp
Gly	Ala, Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Tyr, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

The types of substitutions selected is preferably, but not necessarily, based on the analysis of the frequencies of amino acid substitutions between homologous proteins of different species, such as that developed by Schulz et al., *Principles of Protein Structure*, Springer-Verlag, 1978, on the analyses of structure-forming potentials developed by Chou and Fasman, Biochemistry, 13, 211 (1974) and Adv. Enzymol., 47, 45-149 (1978), and on the analysis of hydrophobicity patterns in proteins developed by Eisenberg et al., Proc. Natl. Acad. Sci. USA, 81, 140-144 (1984); Kyte & Doolittle, J. Molec. Biol., 157, 105-132 (1981), and Goldman et al., Ann. Rev. Biophys. Chem., 15, 321-353 (1986).

GlyT DNAs that encode proteins that exhibit overall less than about 45% sequence identity with each of the proteins encoded by

SEQ ID NOs:1-4 are nonetheless included as GlyT DNA to the extent that the related nucleic acid includes nucleotide and amino acid sequences specific to the genes that encode GlyT-1 or GlyT-2 or substantial portions thereof. By "substantial portions" it is intended that the included portion includes a continuous segment of at least about 50 nucleotides that encode a peptide sequence that exhibits at least about 80% amino acid sequence identity with the corresponding segment of the protein encoded by a glycine transporter nucleic acid sequence, such as but not limited to, SEQ ID NOs:1, 2, 3, or 4; more preferredly, the substantial portion includes a continuous segment of at least about 500 nucleotides that encode a peptide sequence that exhibits at least about 70% amino acid sequence identity with the corresponding segment of the protein encoded by a glycine transporter nucleic acid sequence, such as but not limited to, SEQ ID NOs:1, 2, 3, or 4; and yet more preferredly, the substantial portion includes a continuous segment of at least about 1000 nucleotides that encode a peptide sequence that exhibits at least about 60% amino acid sequence identity with the corresponding segment of the protein encoded by a glycine transporter nucleic acid sequence, such as but not limited to, SEQ ID NOs:1, 2, 3, or 4.

As used in the context of the present invention, the specified sequence identity of a nucleic acid with respect to one of SEQ ID NOs:1-4, or substantial portions thereof, in part defines one embodiment of the GlyT DNA used to generate inventive gene constructs, vectors, and transformed hosts that can be used in the drug discovery method disclosed herein. Accordingly, the nucleic acid used in the context of the present invention is sequenced or otherwise suitably analyzed so as to compare its sequence to one of those of SEQ ID NO: 1, 2, 3, or 4.

Numerous methods for determining percent sequence identity are known in the art. One preferred method is to use version 6.0 of the GAP computer program for making sequence comparisons. The program is available from the University of Wisconsin Genetics Computer Group and utilizes the alignment method of Needleman and Wunsch, J. Mol. Biol., 48, 443, 1970 as revised by Smith and Waterman, Adv. Appl. Math., 2, 482, 1981. Another available method uses the FASTA computer program (Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 85, 2444-2448 (1988)).

As noted above, the present invention relates to cells transfected with GlyT DNA, which is any suitable DNA that encodes a glycine transporter such that glycine transporter properties are expressed by the transfected cells. In one embodiment, such GlyT DNA is homologous to at least one of SEQ ID NOs:1-4, or a sequence complementary thereto; a preferred GlyT DNA of this embodiment encodes a protein that has at least about 45% sequence identity with respect to at least one of SEQ ID NOs:1-4. A more preferred GlyT DNA used in the context of the present invention comprises a nucleic acid selected from the group consisting of SEQ ID NOs:1-4, a nucleic acid complementary thereto, and a substantially equivalent nucleic acid. Such related GlyT DNAs as defined hereinabove are isolated using one of the SEQ ID NOs: 1-4, or substantial portions thereof, as a probe in any of a variety of conventional procedures of molecular biology, including but not limited to hybridization, PCR, or others, on genomic DNA or cDNA derived from organisms that have glycine transport activity, or on genomic or cDNA libraries derived from such organisms.

A "substantially equivalent" nucleic acid is a nucleic acid having a sequence that varies from on of SEQ ID NOs:1-4 by one or more substitutions, deletions, or additions, the effect of which does not result

in an undesirable functional dissimilarity between the two nucleic acids. In other words, the polypeptide that results from the substantially equivalent sequence has the activity characteristic of the GlyT gene product. A difference in sequence at the amino acid level is understood to include amino acid differences, which range from a single amino acid substitution, deletion, or insertion to a number of amino acid substitutions, deletions, and/or insertions, wherein the resulting polypeptide is still recognizable as related to the GlyT protein in that functionality of the glycine transporter is preserved.

A method for the analysis or screening of an agent for treatment of a disease or condition associated with a nervous system disorder or condition comprises culturing separately first and second non-mammalian cells, wherein the first and second non-mammalian cells are preferably of the same species, more preferably of the same strain thereof, and comprise an exogenous nucleic acid encoding a glycine transporter as described herein, preferably either GlyT-1 or GlyT-2, wherein GlyT-1 is preferably GlyT-1a, GlyT-1b, or GlyT-1c. The nervous system disorders or conditions for which the agent can be used for treatment include, but are not limited to, spasticity, myoclonas, muscle spasm, pain, muscle hyperactivity, epilepsy, stroke, head trauma, neuronal cell death, cognitive or memory disorders, multiple sclerosis, spinal cord injury, dystonia, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, attention deficit disorders, organic brain syndromes, and schizophrenia. In this method, the first non-mammalian cell is contacted with the agent, which is preferably a compound, such as a peptide or an organic compound, or a composition or mixture comprising same, as further discussed below, in the presence of a suitably-labeled glycine. Such a labeled glycine has incorporated into it, for example, a radioisotope, such as ^{3}H or ^{14}C. The

contacted first non-mammalian cell is then tested for enhancement or inhibition of glycine transport into the first non-mammalian cell as compared to glycine transport into the second non-mammalian cell that was not contacted with the compound (i.e., the control cell). Such analysis or screening preferably includes activities of finding, learning, discovering, determining, identifying, or ascertaining.

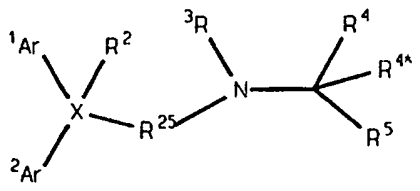
An agent is an enhancer of glycine transport uptake if at the end of the aforestated test the amount of intracellular labeled glycine is greater in the agent-contacted non-mammalian cell than in the non-agent-contacted non-mammalian cell; conversely, an agent is an inhibitor of glycine transport if the amount of intracellular labeled glycine is greater in the non-agent-contacted non-mammalian cell as compared to the other. Preferably, the difference in glycine uptake between the tested first cell and the control second cell is at least about a factor of two; more preferably, the difference is at least about a factor of five; most preferably, the difference is at least about an order of magnitude or greater.

Agents identified using the inventive method are specific for GlyT1, which is preferably GlyT-1a, GlyT-1b, or GlyT-1c, or GlyT-2, or any combination thereof. The same compound preferably is an inhibitor or an enhancer with respect to any one glycine transporter, but may have a neutral or opposite effect with another glycine transporter. Preferred agents have specificity to enhance or inhibit one glycine transporter and have neutral or negligible effect on other glycine transporters as compared to the effect on the indicated glycine transporter. Preferably, an agent having specificity for one glycine transporter with respect to a second glycine transporter has at least an order of magnitude greater potency for inhibiting or activating glycine uptake mediated by the first glycine transporter as compared to its

effect on the second glycine transporter, as tested in transfected cells of the present invention. More preferred agents have differences in potency of at least two orders of magnitude for one glycine transporter as compared to the other.

An agent can be any suitable compound, material, composition, mixture, or chemical, including but not limited to polypeptides of two up to about 25 amino acids in length, preferably from two to about ten, more preferably from two to about five amino acids in length. Other suitable agents in the context of the present invention include small organic compounds, of molecular weight between about 100 daltons and about 5,000 daltons, and are composed of alkyls, aryls, alkenes, alkynes, and other suitable groups, including heteroatoms or not. Such organic compounds can be carbohydrates, including simple sugars, amino or imino acids, nucleic acids, steroids, and others. The chemicals tested as agents hereby may be prepared using combinatorial chemical processes known in the art or conventional means for chemical synthesis. Preferably, suitable agents are useful as drugs for treatment of the aforementioned or other nervous system disorders or conditions.

Agents identified that enhance or inhibit the glycine transporter, or inhibit or activate the glycine receptor portion of the NMDA receptor, using the methods described herein, include those wherein the agent is of the formula:



or a pharmaceutically acceptable salt thereof, wherein:

(1) X is nitrogen or carbon;

(2) Ar^1 is aryl, heteroaryl, arylalkyl wherein the alkyl is C1 to C2, or heteroarylalkyl wherein the alkyl is C1 to C2, and Ar^2 is aryl, heteroaryl, aryloxy, heteroaryloxy, arylalkyl wherein the alkyl is C1 to C2, heteroarylalkyl wherein the alkyl is C1 to C2, arylmethoxy, heteroarylmethoxy, arylthio, heteroarylthio, arylmethylthio, heteroarylmethylthio, or either Ar-N(R^6)- or Ar-CH_2-N($R^{6'}$)-, wherein R^6 and $R^{6'}$ are hydrogen or (C1-C6) alkyl and Ar is aryl or heteroaryl,

(a) wherein when X is nitrogen Ar^2 is not aryloxy, heteroaryloxy, arylmethoxy, heteroarylmethoxy, arylthio, heteroarylthio, arylmethylthio, heteroarylmethylthio, Ar-N(R^6)- or Ar-CH_2-N($R^{6'}$)-,

(b) wherein the aryl of Ar^1 or Ar^2 is phenyl or naphthyl,

(c) wherein the heteroaryl of Ar^1 or Ar^2 comprises a five-membered ring, a six-membered ring, a six-membered ring fused to a five-membered ring, or a six-membered ring fused to a six-membered ring, wherein the heteroaryl is aromatic and contains heteroatoms selected from the group consisting of oxygen, sulfur and nitrogen, with the remaining ring atoms being carbon,

(d) wherein the aryl or heteroaryl of Ar^1 and Ar^2 together can be substituted with up to six substituents selected from the group consisting of fluoro, chloro, bromo, nitro, cyano, trifluoromethyl, amidosulfonyl which can have up to two (C1-C6) N-alkyl substitutions, (C1-C6) alkyl, (C2-C6) alkenyl, amino, (C1-C6) alkylamino, dialkylamino wherein each alkyl is independently C1 to C6, (C1-C6) alkoxy, (C2-C7) alkanoyl, (C2-C7) alkanoyloxy, trifluoromethoxy, hydroxycarbonyl, (C2-C7)

alkyloxycarbonyl, aminocarbonyl that can be substituted for hydrogen with up to two (C1-C6) alkyl, (C1-C6) alkylsulfonyl, amidino that can independently substituted with up to three (C1-C6) alkyl, or methylenedioxy or ethylenedioxy with the two oxygens bonded to adjacent positions on the aryl or heteroarly ring structure, which methylenedioxy or ethylenedioxy can be substituted with up to two (C1-C6) alkyl,

(i.) wherein such substitutions to the aryl or heteroaryl of Ar^1 and Ar^2 can be combined to form a second bridge between Ar^1 and Ar^2 comprising **(1)** (C1-C2) alkyl or alkenyl, which can be independently substituted with one or more (C1-C6) alkyl, **(2)** sulfur, **(3)** oxygen, **(4)** amino, which can be substituted for hydrogen with one (C1-C6) alkyl, **(5)** carbonyl, **(6)** $-CH_2C(=O)-$, which can be substituted for hydrogen with up to two (C1-C6) alkyl, **(7)** $-C(=O)-O-$, **(8)** $-CH_2-O-$, which can be substituted for hydrogen with up to two (C1-C6) alkyl, **(9)** $-C(=O)-N(R^{24})-$, wherein R^{24} is hydrogen or (C1-C6) alkyl, **(10)** $-CH_2-NH-$, which can be substituted for hydrogen with up to three (C1-C6) alkyl, **(11)** -CH=N-, which can be substituted for hydrogen with (C1-C6) alkyl, or wherein the aryls or heteroaryls of Ar^1 and Ar^2 can be directly linked by a single bond;

(3) R^{25} comprises **(a)** a straight-chained (C1-C4) aliphatic group, **(b)** $=N-O-(R^{26})$ when X is carbon, wherein R^{26} is ethylene or propylene and the unmatched double bond is linked to X, or **(c)** $-0-R^8$ or $-S-R^{8'}$ when X is carbon and Ar^2 is neither $Ar-N(R^6)-$ nor $Ar-CH_2-N(R^{6'})-$, wherein R^8 or $R^{8'}$ is a (C2-C3) alkylene or (C2-C3) alkenylene and O or S is bonded to X,

(i.) wherein R^{25} can be substituted with up to one hydroxy, up to one (C1-C6) alkoxy or up to one (C2-C7) alkanoyloxy, with up to two (C1-C6) alkyl, with up to one oxo, up to one (C1-C6) alkylidene, with the proviso that the hydroxy, alkoxy, alkanoyloxy or oxo substituents are not bonded to a carbon that is bonded to a nitrogen or oxygen,

(ii.) wherein the alkyl or alkylidene substituents of R^{25} can be linked to form a 3 to 7-membered ring,

(iii.) wherein if X is nitrogen, X is linked to R^{25} by a single bond and the terminal carbon of R^{25} that links R^{25} to N is saturated;

(4) R^2 **(a)** is not present when X is nitrogen, **(b)** is hydrogen, (C1-C6) alkyl, (C1-C6) alkoxy, cyano, (C2-C7) alkanoyl, aminocarbonyl, (C1-C6) alkylaminocarbonyl, dialkylaminocarbonyl wherein each alkyl is independently C1-C6, or Ar^9 where Ar^9 is independently as defined for Ar^1, **(c)** comprises, where R^{25} is not -O-R^8, hydroxy, fluoro, chloro, bromo or (C2-C7) alkanoyloxy, **(d)** forms a double bond with an adjacent carbon or nitrogen from R^{25};

(5) R^3 **(a)** is hydrogen, (C1-C6) alkyl, or phenyl or phenylalkyl wherein the alkyl is C1-C6 and either such phenyl can be substituted with up to 3 of the same substituents defined above for the aryl or heteroaryl of Ar^1 or Ar^2, **(b)** is -$CH(R^9)$-R^{10}, wherein R^9 is the same as R^4 and R^{10} is the same as R^5, or **(c)** $Z(Ar^3)(Ar^4)(R^{11})$-R^{12}, wherein R^{12} is bonded to N, Z is independently the same as X, Ar^3 is independently the same as Ar^1, Ar^4 is independently the same as Ar^2, R^{11} is independently the same as R^2 and R^{12} is independently the same as R^{25};

(6) R^4 and $R^{4'}$ are independently hydrogen or (C1-C6) alkyl that can be bonded to complete a 3 to 7-membered ring, or one of R^4 and $R^{4'}$ can be (C1-C6) hydroxyalkyl; and

(7) R^5 is $(CO)NR^{13}R^{14}$, $(CO)OR^{15}$, $(CO)SR^{16}$, $(SO_2)NR^{17}R^{18}$, $(PO)(OR^{19})(OR^{20})$ or CN, wherein R^{13}, R^{14}, R^{15}, R^{16} R^{17}, R^{18} R^{19} and R^{20} are independently hydrogen, (C1-C8) alkyl which can incorporate a (C3-C8) cycloalkyl, wherein the carbon linked to the oxygen of R^{15} or the sulfur of R^{16} has no more than secondary branching and , (C2-C6) hydroxyalkyl, aminoalkyl where the alkyl is C2-C6 and the amino can be substituted with up to two (C1-C6) alkyls, arylalkyl wherein the alkyl is C1 to C6, heteroarylalkyl wherein the alkyl is C1 to C6, aryl or heteroaryl,

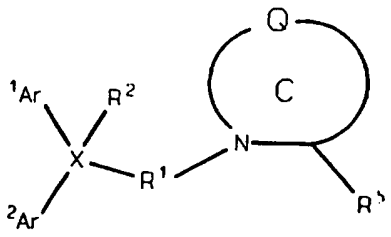
(a) wherein the aryl is phenyl or napthyl and the heteroaryl is a five-membered ring, a six-membered ring, a six-membered ring fused to a five-membered ring, or a six-membered ring fused to a six-membered ring, wherein the heteroaryl is aromatic and contains heteroatoms selected from the group consisting of oxygen, sulfur and nitrogen, with the remaining ring atoms being carbon,

(b) wherein the aryl, heteroaryl, aryl or arylalkyl or the heteroaryl of heteroarylalkyl can be substituted with up to three substituents selected from the group consisting of fluoro, chloro, bromo, nitro, cyano, trifluoromethyl, amidosulfonyl which can have up to two (C1-C6) N-alkyl substitutions, (C1-C6) alkyl, (C2-C6) alkenyl, (C1-C6) alkylamine, dialkylamine wherein each alkyl is independently C1 to C6, amino, (C1-C6) alkoxy, (C2-C7) alkanoyl, (C2-C7) alkanoyloxy, trifluoromethoxy, hydroxycarbonyl, (C2-C7) alkyloxycarbonyl, aminocarbonyl

that can be N-substituted with up to two (C1-C6) alkyl, (C1-C6) alkylsulfonyl, amidino that can substituted with up to 3 (C1-C6) alkyl, or methylenedioxy or ethylenedioxy with the two oxygens bonded to adjacent positions on the aryl or heteroaryl ring structure, which methylenedioxy or ethylenedioxy can be substituted with up to two (C1-C6) alkyl, and

(c) wherein R^{13} and R^{14} together with the nitrogen can form a 5 to 7-membered ring that can contain one additional heteroatom selected from oxygen and sulfur.

Other suitable agents identified as above include those wherein the agent is of the formula:



or a pharmaceutically acceptable salt thereof, wherein:

(1) X is nitrogen or carbon;

(2) Ar^1 is aryl, heteroaryl, arylalkyl wherein the alkyl is C1 to C2, or heteroarylalkyl wherein the alkyl is C1 to C2, and Ar^2 is aryl, heteroaryl, aryloxy, heteroaryloxy, arylalkyl wh rein the alkyl is C1 to C2, h teroarylalkyl wherein the alkyl is C1 to C2, arylmethoxy,

heteroarylmethoxy, arylthio, heteroarylthio, arylmethylthio, heteroarylmethylthio, or either Ar-N(R^6)- or Ar-CH_2-N($R^{6'}$)-, wherein R^6 and $R^{6'}$ are hydrogen or (C1-C6) alkyl and Ar can be aryl or heteroaryl,

(a) wherein when X is nitrogen Ar^2 is not aryloxy, heteroaryloxy, arylmethoxy, heteroarylmethoxy, arylthio, heteroarylthio, arylmethylthio, heteroarylmethylthio, Ar-N(R^6)- or Ar-CH_2-N($R^{6'}$)-,

(b) wherein the aryl of Ar^1 or Ar^2 is phenyl or naphthyl,

(c) wherein the heteroaryl of Ar^1 or Ar^2 comprises a five-membered ring, a six-membered ring, a six-membered ring fused to a five-membered ring, or a six-membered ring fused to a six-membered ring, wherein the heteroaryl is aromatic and contains heteroatoms selected from the group consisting of oxygen, sulfur and nitrogen, with the remaining ring atoms being carbon,

(d) wherein the aryl or heteroaryl of Ar^1 and Ar^2 together can be substituted with up to six substituents selected from the group consisting of fluoro, chloro, bromo, nitro, cyano, trifluoromethyl, amidosulfonyl which can have up to two (C1-C6) N-alkyl substitutions, (C1-C6) alkyl, (C2-C6) alkenyl, amino, (C1-C6) alkylamino, dialkylamino wherein each alkyl is independently C1 to C6, (C1-C6) alkoxy, (C2-C7) alkanoyl, (C2-C7) alkanoyloxy, trifluoromethoxy, hydroxycarbonyl, (C2-C7) alkyloxycarbonyl, aminocarbonyl that can be substituted for hydrogen with up to two (C1-C6) alkyl, (C1-C6) alkylsulfonyl, amidino that can independently substituted for hydrogen with up to three (C1-C6) alkyl, or methylenedioxy or ethylenedioxy with the two oxygens bonded to adjacent positions on the aryl or

heteroaryl ring structure, which methylenedioxy or ethylenedioxy can be substituted with up to two (C1-C6) alkyl,

(i.) wherein such substitutions to the aryl or heteroaryl of Ar^1 and Ar^2 can be combined to form a second bridge between Ar^1 and Ar^2 comprising **(1)** (C1-C2) alkyl or alkenyl, which can be independently substituted with one or more (C1-C6) alkyl, **(2)** sulfur, **(3)** oxygen, **(4)** amino, which can be substituted for hydrogen with one (C1-C6) alkyl, **(5)** carbonyl, **(6)** $-CH_2C(=O)-$, which can be substituted for hydrogen with up to two (C1-C6) alkyl, **(7)** $-C(=O)-O-$, **(8)** $-CH_2-O-$, which can be substituted for hydrogen with up to two (C1-C6) alkyl, **(9)** $-C(=O)-N(R^{24})-$, wherein R^{24} is hydrogen or (C1-C6) alkyl, **(10)** $-CH_2-NH-$, which can be substituted for hydrogen with up to three (C1-C6) alkyl, or **(11)** $-CH=N-$, which can be substituted for hydrogen with (C1-C6) alkyl, or wherein the aryls or heteroaryls of Ar^1 and Ar^2 can be directly linked by a single bond;

(3) R^1 comprises **(a)** a straight-chained (C2-C4) aliphatic group, **(b)** $=N-O-(CH_2CH_2)-$ when X is carbon, wherein the unmatched double bond is linked to X, or **(c)** $-O-R^8$ or $-S-R^{8*}-$ when X is carbon and Ar^2 is neither $Ar-N(R^6)-$ nor $Ar-CH_2-N(R^{6*})-$, wherein R^8 or R^{8*} is a (C2-C3) alkylene or (C2-C3) alkenylene and O or S is bonded to X,

(i.) wherein R^1 can be substituted with up to one hydroxy, up to one (C1-C6) alkoxy or up to one (C2-C7) alkanoyloxy, with up to two (C1-C6) alkyl, with up to one oxo, up to one (C1-C6) alkylidene,

with the proviso that the hydroxy, alkoxy, alkanoyloxy or oxo substituents are not bonded to a carbon that is bonded to a nitrogen or oxygen,

(ii.) wherein the alkyl or alkylidene substituents of R^1 can be linked to form a 3 to 7-membered ring,

(iii.) wherein if X is nitrogen, X is linked to R^1 by a single bond and wherein the terminal carbon of R^1 that links R^1 to N is saturated;

(4) R^2 **(a)** is not present when X is nitrogen, **(b)** is hydrogen, (C1-C6) alkyl, (C1-C6) alkoxy, cyano, (C2-C7) alkanoyl, aminocarbonyl, (C1-C6) alkylaminocarbonyl or dialkylaminocarbonyl wherein each alkyl is independently C1 to C6, **(c)** comprises, where R^1 is not -O-R^8 or -S-R^{8*}-, hydroxy, fluoro, chloro, bromo or (C2-C7) alkanoyloxy, **(d)** forms a double bond with an adjacent carbon or nitrogen from R1;

(5) wherein Q together with the illustrated tertiary nitrogen and tertiary carbon bearing R^5 form ring **C**, wherein ring **C** is a 3 to 8-membered ring, a 3 to 8-membered ring substituted with a 3 to 6-membered spiro ring, or a 3 to 8-membered ring fused with a 5 to 6-membered ring, wherein the fused ring lacking the illustrated tertiary nitrogen can be aromatic or heteroaromatic, wherein for each component ring of ring **C** there are up to two heteroatoms selected from oxygen, sulfur or nitrogen, including the illustrated nitrogen, and the rest carbon, with the proviso that the ring atoms include no quaternary nitrogens, with the proviso that, in saturated rings, ring nitrogen atoms are separated from other ring heteroatoms by at least two intervening carbon atoms,

(a) wherein the carbon and nitrogen ring atoms of ring **C** can be substituted with up to three substituents selected from (C1-C6) alkyl, (C2-C6) alkenylene, cyano, nitro, trifluoromethyl, (C2-

C7) alkyloxycarbonyl, (C1-C6) alkylidene, hydroxyl, (C1 - C6) alkoxy, oxo, hydroxycarbonyl, aryl wherein the aryl is as defined for Ar^1 or heteroaryl wherein the heteroaryl is as defined for Ar^1, with the proviso that ring atoms substituted with alkylidene, hydroxycarbonyl or oxo are carbon, with the further proviso that ring atoms substituted with hydroxyl or alkoxy are speparated from other ring heteroatoms by at least two intervening carbon atoms,

(b) and wherein Q is as appropriate to satisfy the definition of ring C; and

(6) R^5 is $(CO)NR^{13}R^{14}$, $(CO)OR^{15}$, $(CO)SR^{16}$, $(SO_2)NR^{17}R^{18}$, $(PO)(OR^{19})(OR^{20})$ or CN, wherein R^{13}, R^{14}, R^{15}, R^{16} R^{17}, R^{18} R^{19} and R^{20} are independently hydrogen; (C1-C8) alkyl which can incorporate a (C3-C8) cycloalkyl, wherein the carbon linked to the oxygen of R^{15} or the sulfur of R^{16} has no more than secondary branching and , (C2-C6) hydroxyalkyl, aminoalkyl where the alkyl is C2 to C6 and the amino can be substituted with up to two (C1-C6) alkyls, arylalkyl wherein the alkyl is C1 to C6, heteroarylalkyl wherein the alkyl is C1 to C6, aryl or heteroaryl,

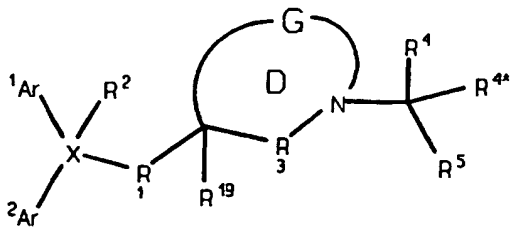
(a) wherein the aryl is phenyl or napthyl and the heteroaryl is a five-membered ring, a six-membered ring, a six-membered ring fused to a five-membered ring, or a six-membered ring fused to a six-membered ring, wherein the heteroaryl is aromatic and contains heteroatoms selected from the group consisting of oxygen, sulfur and nitrogen, with the remaining ring atoms being carbon,

(b) wherein the aryl, heteroaryl, aryl or arylalkyl or the heteroaryl of heteroarylalkyl can be substituted with up to three substituents selected from the group consisting of

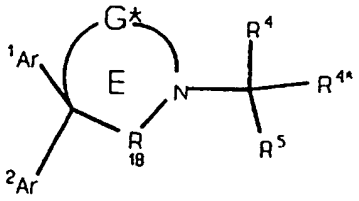
fluoro, chloro, bromo, nitro, cyano, trifluoromethyl, amidosulfonyl which can have up to two (C1-C6) N-alkyl substitutions, (C1-C6) alkyl, (C2-C6) alkenyl, (C1-C6) alkylamine, dialkylamine wherein each alkyl is independently C1 to C6, amino, (C1-C6) alkoxy, (C2-C7) alkanoyl, (C2-C7) alkanoyloxy, trifluoromethoxy, hydroxycarbonyl, (C2-C7) alkyloxycarbonyl, aminocarbonyl that can be N-substituted with up to two (C1-C6) alkyl, (C1-C6) alkylsulfonyl, amidino that can substituted for hydrogen with up to three (C1-C6) alkyl, or methylenedioxy or ethylenedioxy with the two oxygens bonded to adjacent positions on the aryl or heteroaryl ring structure, which methylenedioxy or ethylenedioxy can be substituted with up to two (C1-C6) alkyl,

(c) wherein R^{13} and R^{14} together with the nitrogen to which they are bonded can form a 5 to 7-membered ring that can contain one additional heteroatom selected from oxygen and sulfur.

Yet other suitable agents identified as above include those wherein the agent is of the following formula I or II:



I



II

or a pharmaceutically acceptable salt thereof, wherein:

(1) X is nitrogen or carbon;

(2) Ar^1 is aryl, heteroaryl, arylalkyl wherein the alkyl is C1 to C2, or heteroarylalkyl wherein the alkyl is C1 to C2, and Ar^2 is aryl, heteroaryl, aryloxy, heteroaryloxy, arylalkyl wherein the alkyl is C1 to C2, heteroarylalkyl wherein the alkyl is C1 to C2, arylmethoxy, heteroarylmethoxy, arylthio, heteroarylthio, arylmethylthio, heteroarylmethylthio, or either Ar-N(R^6)- or Ar-CH_2-N(R^{6*})-, wherein R^6 and R^{6*} are hydrogen or (C1-C6) alkyl and Ar can be aryl or heteroaryl,

(a) wherein when X is nitrogen Ar^2 is not aryloxy, heteroaryloxy, arylmethoxy, heteroarylmethoxy, arylthio, heteroarylthio, arylmethylthio, heteroarylmethylthio, Ar-N(R^6)- or Ar-CH_2-N(R^{6*})-;

(b) wherein the aryl of Ar^1 or Ar^2 is phenyl or naphthyl,

(c) wherein the heteroaryl of Ar^1 or Ar^2 comprises a five-membered ring, a six-membered ring, a six-membered ring fused to a five-membered ring, or a six-membered ring fused to a six-membered ring, wherein the heteroaryl is aromatic and contains heteroatoms selected from the group consisting of oxygen, sulfur and nitrogen, with the remaining ring atoms being carbon,

(d) wherein the aryl or heteroaryl of Ar^1 and Ar^2 together can be substituted with up to six substituents selected from the group consisting of fluoro, chloro, bromo, nitro, cyano, trifluoromethyl, amidosulfonyl which can have up to two (C1-C6) N-alkyl substitutions, (C1-C6) alkyl, (C2-C6) alkenyl, amino, (C1-C6) alkylamino, dialkylamino wherein each alkyl is independently C1 to C6, (C1-C6) alkoxy, (C2-C7) alkanoyl, (C2-C7) alkanoyloxy, trifluoromethoxy, hydroxycarbonyl, (C2-C7) alkyloxycarbonyl, aminocarbonyl that can be substituted for hydrogen with up to two (C1-C6) alkyl, (C1-C6) alkylsulfonyl, amidino that can independently substituted for hydrogen with up to three (C1-C6) alkyl, or methylenedioxy or ethylenedioxy with the two oxygens bonded to adjacent positions on the aryl or heteroaryl ring structure, which methylenedioxy or ethylenedioxy can be substituted with up to 2 (C1-C6) alkyl,

(i.) wherein such substitutions to the aryl or heteroaryl of Ar^1 and Ar^2 can be combined to form a second bridge between Ar^1 and Ar^2 comprising **(1)** (C1-C2) alkyl or alkenyl, which can be substituted with one or more (C1-C6) alkyl, **(2)** sulfur, **(3)** oxygen, **(4)** amino, which can be substituted for hydrogen with one (C1-C6) alkyl, **(5)** carbonyl, **(6)** $-CH_2C(=O)-$, which can be substituted for hydrogen with up to two (C1-C6) alkyl, **(7)** $-C(=O)-O-$, **(8)** $-CH_2-O-$, which can be substituted for hydrogen with up to two (C1-C6) alkyl, **(9)** $-C(=O)-N(R^{24})-$, wherein R^{24} is hydrogen or (C1-C6) alkyl, **(10)** $-CH_2-NH-$, which can be substituted for hydrogen with up to three

(C1-C6) alkyl, or **(11)** -CH=N-, which can be substituted for hydrogen with (C1-C6) alkyl, or wherein the aryls or heteroaryls of Ar^1 and Ar^2 can be directly linked by a single bond;

(3) R^1 comprises **(a)** a single bond or double bond, **(b)** a straight-chained (C1-C3) aliphatic group, **(c)** =N-O-(CH_2CH_2)- when X is carbon, wherein the unmatched double bond is linked to X, or **(d)** -0-R^8 or -S-$R^{8'}$ when X is carbon and Ar^2 is neither Ar-N(R^6)- nor Ar-CH_2-N($R^{6'}$)-, wherein either R^8 or $R^{8'}$ is a single bond, (C1-C3) alkylene or (C2-C3) alkenylene and O or S is bonded to X,

(i.) wherein R^1 can be substituted with up to one hydroxy, up to one (C1-C6) alkoxy or up to one (C2-C7) alkanoyloxy, with up to two (C1-C6) alkyl, with up to one oxo, up to one (C1-C6) alkylidene, with the proviso that the hydroxy, alkoxy, alkanoyloxy or oxo substituents are not bonded to a carbon that is bonded to a nitrogen or oxygen,

(ii.) wherein the alkyl or alkylidene substituents of R^1 can be linked to form a 3 to 7-membered ring,

(iii.) wherein if X is nitrogen, X is linked to R^1 by a single bond and the terminal carbon of R^1 that links R^1 to N is saturated;

(4) R^2 **(a)** is not present when X is nitrogen, **(b)** is hydrogen, (C1-C6) alkyl, (C1-C6) alkoxy, cyano, (C2-C7) alkanoyl, aminocarbonyl, (C1-C6) alkylaminocarbonyl or dialkylaminocarbonyl wherein each alkyl is independently C1-C6, **(c)** comprises, where R^1 is not -O-R^8, hydroxy, fluoro, chloro, bromo or(C2-C7) alkanoyloxy, **(d)** forms a double bond with an adjacent carbon or nitrogen from R1;

(5) R^3 is a single bond or (C1-C2) alkyl or alk nyl;

(6) R^{18} is a single bond or (C1-C3) alkyl or alkenyl;

(7) wherein ring **D** is a 3 to 8-membered ring, a 3 to 8-membered ring substituted with a 3 to 6-membered spiro ring, or a 3 to 8-membered ring fused with a 5 to 6-membered ring, wherein the fused ring lacking the illustrated tertiary nitrogen can be aromatic or heteroaromatic, wherein for each component ring of ring **D** there are up to two heteroatoms selected from oxygen, sulfur or nitrogen, including the illustrated nitrogen, and the rest carbon, with the proviso that the ring atoms include no quaternary nitrogens, with the proviso that, in saturated rings, ring nitrogen atoms are separated from other ring heteroatoms by at least two intervening carbon atoms,

(a) wherein the carbon and nitrogen ring atoms of ring **D** can be substituted with up to three substituents selected from (C1-C6) alkyl, (C2-C6) alkenylene, cyano, nitro, trifluoromethyl, (C2-C7) alkyloxycarbonyl, (C1-C6) alkylidene, hydroxyl, (C1-C6) alkoxy, oxo, hydroxycarbonyl, aryl wherein the aryl is as defined for Ar^1 or heteroaryl wherein the heteroaryl is as defined for Ar^1, with the proviso that ring atoms substituted with alkylidene, hydroxycarbonyl or oxo are carbon, with the further proviso that ring atoms substituted with hydroxyl or alkoxy are separated from other ring heteroatoms by at least two intervening carbon atoms,

(b) and wherein G is a required to satisfy the definition of ring **D**;

(8) wherein ring **E** is a 3 to 8-membered ring, a 3 to 8-membered ring substituted with a 3 to 6-membered spiro ring, or a 3 to 8-membered ring fused with a 5 to 6-membered ring, wherein the fused ring lacking the illustrated tertiary nitrogen can be aromatic or heteroaromatic, wherein for each component ring of ring **E** there are up

to two heteroatoms selected from oxygen, sulfur or nitrogen, including the illustrated nitrogen, and the rest carbon, with the proviso that the ring atoms include no quaternary nitrogens, with the proviso that, in saturated rings, ring nitrogen atoms are separated from other ring heteroatoms by at least two intervening carbon atoms,

(a) wherein the carbon and nitrogen ring atoms of ring **E** can be substituted with up to three substituents selected from (C1-C6) alkyl, (C2-C6) alkenylene, cyano, nitro, trifluoromethyl, (C2-C7) alkyloxycarbonyl, (C1-C6) alkylidene, hydroxyl, (C-C6) alkoxy, oxo, hydroxycarbonyl, (C1-C6) alkoxycarbonyl, aryl wherein the aryl is as defined for Ar^1 or heteroaryl wherein the heteroaryl is as defined for Ar^1, with the proviso that ring atoms substituted with alkylidene, hydroxycarbonyl or oxo are carbon, with the further proviso that ring atoms substituted with hydroxyl or alkoxy are separated from other ring heteroatoms by at least two intervening carbon atoms;

(b) and wherein G^* is a required to satisfy the definition of ring **E**;

(9) R^{19} **(a)** forms a double bond with R^1, R^3 or G, **(b)** is hydrogen **(c)** is (C1 - C3) alkyl or alkylene, or **(d)** is incorporporated into a fused ring;

(10) R^4 and $R^{4'}$ are independently hydrogen or (C1-C6) alkyl that can be bonded to complete a 3 to 7-membered ring, or one of R^4 and $R^{4'}$ can be (C1-C6) hydroxyalkyl; and

(11) R^5 is $(CO)NR^{13}R^{14}$, $(CO)OR^{15}$, $(CO)SR^{16}$, $(SO_2)NR^{17}R^{18}$, $(PO)(OR^{21})(OR^{20})$ or CN, wherein R^{13}, R^{14}, R^{15}, R^{16} R^{17}, R^{18}, R^{21} and R^{20} are independently hydrogen, (C1-C8) alkyl which can incorporate a (C3-C8) cycloalkyl, wherein the carbon linked to the oxygen of R^{15} or the sulfur of R^{16} has no more than secondary branching and , (C2-C6)

hydroxyalkyl, aminoalkyl where the alkyl is C2 to C6 and the amino can be substituted with up to two (C1-C6) alkyls, arylalkyl wherein the alkyl is C1 to C6, heteroarylalkyl wherein the alkyl is C1 to C6, aryl or heteroaryl,

(a) wherein the aryl is phenyl or napthyl and the heteroaryl is a five-membered ring, a six-membered ring, a six-membered ring fused to a five-membered ring, or a six-membered ring fused to a six-membered ring, wherein the heteroaryl is aromatic and contains heteroatoms selected from the group consisting of oxygen, sulfur and nitrogen, with the remaining ring atoms being carbon,

(b) wherein the aryl, heteroaryl, aryl or arylalkyl or the heteroaryl of heteroarylalkyl can be substituted with up to three substituents selected from the group consisting of fluoro, chloro, bromo, nitro, cyano, trifluoromethyl, amidosulfonyl which can have up to two (C1-C6) N-alkyl substitutions, (C1-C6) alkyl, (C2-C6) alkenyl, (C1-C6) alkylamine, dialkylamine wherein each alkyl is independently C1 to C6, amino, (C1-C6) alkoxy, (C2-C7) alkanoyl, (C2-C7) alkanoyloxy, trifluoromethoxy, hydroxycarbonyl, (C2-C7) alkyloxycarbonyl, aminocarbonyl that can be N-substituted with up to two (C1-C6) alkyl, (C1-C6) alkylsulfonyl, amidino that can substituted with up to three (C1-C6) alkyl, or methylenedioxy or ethylenedioxy with the two oxygens bonded to adjacent positions on the aryl or heteroaryl ring structure, which methylenedioxy or ethylenedioxy can be substituted with up to two (C1-C6) alkyl, and

(c) wherein R^{13} and R^{14} together with the nitrogen can form a 5 to 7-membered ring that can contain one additional heteroatom selected from oxygen and sulfur.

Some compounds that inhibit GlyT-1 or GlyT-2 mediated transport also bind to the glycine binding site on the NMDA receptor. Such binding can be identified by a binding assay whereby, for example, radiolabelled glycine is placed in contact with a preparation of NMDA receptors, such as can be prepared from neuronal cells or brain tissue. See, for example, Grimwood et al., Molec. Pharmacol., 41, 923-930 (1992). In particular, one can prepare such NMDA receptors by isolating a membrane fraction from selected brain tissue of a suitable animal. Suitable brain tissue includes, but is not limited to, cortices and hippocampi, as isolated from any mammal. A membrane fraction can be prepared therefrom using conventional means, and includes, for example, methods of homogenization and centrifugation. The NMDA receptor located in such membranes is treated using mild detergent, such as about 0.1% to about 0.5% saponin, to remove any endogenous glycine or glutamate. The glycine used in such an assay is radiolabelled with any suitable isotope, such as ^{14}C or ^{3}H.

Specific binding of the radiolabelled glycine is then determined by subtracting the quantified radioactivity due to non-specific binding from that which is due to total (*i.e.*, specific and non-specific) binding of the radiolabelled glycine. The radioactivity due to non-specific binding is determined by quantifying the amount of radiolabel associated with an NMDA receptor-containing membrane fraction that has been contacted with radiolabelled glycine and with at least a 100-fold excess of non-radiolabelled or "cold" glycine. The radioactivity due to total binding of the radiolabelled glycine is determined by quantifying the amount of radiolabel bound to the NMDA

receptor preparation in the absence of non-radiolabeled glycine. One can also measure binding to the glycine site on the NMDA receptor using labeled analogs of amino acids, such as, for example, dichlorokynurenic acid or L-689,560. See Grimwood et al., Molecular Pharmacol., 49, 923-930 (1992).

Another way to measure binding of a compound to the glycine site on the NMDA receptor is by measuring the compound's ability to modulate the binding of [^{3}H]MK-801 to the NMDA receptor. MK-801 binds to the NMDA receptor at a different site than does glycine, but binding of glycine or other ligands to the glycine site can allosterically modulate the binding of MK-801. An advantage of this technique is that it allows one to distinguish compounds having agonist activity from those having antagonist activity at the NMDA-receptor-glycine binding site. In particular, compounds having agonist activity in this assay enhance MK-801 binding; conversely, compounds having antagonist activity inhibit MK-801 binding. Sterner and Calligaro, Soc. Neurosci. Abstr., 21, 351 (1995); Calligaro et al., J. Neurochem., 60, 2297-2303 (1993).

A functional ion-flux assay used to measure the effect of compounds identified by the present invention relates to the ability to enhance or inhibit calcium flux through the NMDA receptor. This test is performed on suitable cell cultures that have membrane-bound NMDA receptors and glycine transporters. Such cells include neuronal cells generally, including those of the central nervous system, including brain, and cell lines derived therefrom, and any other cell that has been induced or transfected to express NMDA receptors. Calcium used in such a test is commonly the ^{45}Ca isotope, although other calcium measuring techniques can be used as well, such as calcium-associated fluorescence and th lik . However the calcium is monitored, calcium

flux is enhanced or inhibited as a result of the discrete addition of a compound of the present invention. An advantage of this system is that it allows one to monitor the net effect on NMDA receptor function of a compound that interacts with the glycine site on the NMDA receptor and the glycine transporter.

GlyT-1 inhibitors that are also NMDA receptor agonists act to alleviate schizophrenia and enhance cognition both by increasing glycine concentrations at the NMDA receptor-expressing synapses via inhibition of the glycine transporter, and via directly enhancing NMDA receptor activity. Glycine transporter inhibitors that are also NMDA receptor antagonists can nonetheless retain activity in schizophrenia and enhancing cognition, if the increase in glycine due to glycine transport inhibition prevails over the NMDA antagonism. Where the NMDA receptor antagonist activity prevails over the effect of increased extracellular glycine resulting from inhibition of the glycine transporter, these compounds are useful in limiting the cell damage and cell death arising after stroke or as a consequence of neurodegenerative diseases such as Alzheimer's, Parkinson's, AIDS dementia, Huntington's, and the like. See, for example, Choi, *supra*; Coyle and Puttfarcken, *supra*; Lipton and Rosenberg, *supra*; Brennan, Chem. Eng. News (May 13, 1996), pp. 41-47; Leeson, in Drug Design For Neuroscience (Alan P. Kozikowski, ed., 1993), pp. 339-383.

As discussed above, the compounds of the invention have a number of pharmacological actions. The relative effectiveness of the compounds can be assessed in a number of ways, including the following:

1. Comparing the activity mediated through GlyT-1 and GlyT-2 transporters. This testing identifies compounds (a) that are more active against GlyT-1 transporters and thus more useful in

treating or preventing schizophrenia, increasing cognition and enhancing memory or (b) that are more active against GlyT-2 transporters and thus more useful in treating or preventing epilepsy, pain or spasticity.

2. Testing for NMDA receptor binding. This test establishes whether there is sufficient binding at this site, whether antagonist or agonist activity, to warrant further examination of the pharmacological effect of such binding.

3. Testing the activity of the compounds in enhancing or diminishing calcium fluxes in primary neuronal tissue culture. A test compound that increases calcium flux either (a) has little or no antagonist activity at the NMDA receptor and should not affect the potentiation of glycine activity through GlyT-1 transporter inhibition or (b), if marked increases are observed over comparison with GlyT-1 inhibitors that have little direct interaction with NMDA receptors, then the compound is a receptor agonist. In either of the above-described cases, the test confirms activity in treating or preventing schizophrenia, increasing cognition, or enhancing memory. In contrast, a test compound that decreases calcium flux has a net effect wherein receptor antagonist activity predominates over any activity the compound has in increasing glycine activity through inhibiting glycine transport. In this case, the test confirms activity in limiting or preventing the cell damage and cell death arising after stroke or other ischemia-inducing conditions, or in limiting or preventing the cell damage associated with neurodegenerative diseases.

The following examples further illustrate the present invention, but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

This example sets forth methods and materials used for growing and transfecting QT-6 cells.

QT-6 cells were obtained from American Type Culture Collection (Accession No. ATCC CRL-1708). Complete QT-6 medium for growing QT-6 is Medium 199 (Sigma Chemical Company, St. Louis, MO; hereinafter "Sigma") supplemented to be 10% tryptose phosphate; 5% fetal bovine serum (Sigma); 1% penicillin-streptomycin (Sigma); and 1% sterile dimethylsulfoxide (DMSO; Sigma). Other solutions required for growing or transfecting QT-6 cells included:

DNA/DEAE Mix: 450 μl TBS, 450 μl DEAE Dextran (Sigma), and 100 μl of DNA (4 μg) in TE, where the DNA includes GlyT-1a, GlyT-1b, GlyT-1c, or GlyT-2, in a suitable expression vector. The DNA used was as defined below.

PBS: Standard phosphate buffered saline, pH 7.4 including 1 mM $CaCl_2$ and 1 mM $MgCl_2$ sterilized through 0.2 μ filter.

TBS: One ml of Solution B, 10 ml of Solution A; brought to 100 ml with distilled H_2O; filter-sterilized and stored at 4°C.

TE: 0.01 M Tris, 0.001 M EDTA, pH 8.0.

DEAE dextran: Sigma, #D-9885. A stock solution was prepared consisting of 0.1% (1 mg/ml) of the DEAE dextran in TBS. The stock solution was filter sterilized and frozen in 1 ml aliquots.

Chloroquine: Sigma, #C-6628. A stock solution was prepared consisting of 100 mM chloroquine in H_2O. The stock solution was filter-sterilized and stored in 0.5 ml aliquots, frozen.

Solution A (10X):

NaCl	8.00 g
KCl	0.38 g
Na_2HPO_4	0.20 g

Tris base 3.00 g

The solution was adjusted to pH 7.5 with HCl, brought to 100.0 ml with distilled H_2O, and filter-sterilized and stored at room temperature.

Solution B (100X):

$CaCl_2 \cdot 2H_2O$	1.5 g
$MgCl_2 \cdot 6H_2O$	1.0 g

The solution was brought to 100 ml with distilled H_2O, and filter-sterilized; the solution was then stored at room temperature.

HBSS: 150 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2 \cdot H_2O$; adjusted with NaOH to pH 7.4.

Standard growth and passaging procedures used were as follows: Cells were grown in 225 ml flasks. For passaging, cells were washed twice with warm HBSS (5 ml each wash). Two ml of a 0.05% trypsin/EDTA solution was added, the culture was swirled, then the trypsin/EDTA solution was aspirated quickly. The culture was then incubated about 2 minutes (until cells lift off), then 10 ml of QT-6 media was added and the cells were further dislodged by swirling the flask and tapping its bottom. The cells were removed and transferred to a 15 ml conical tube, centrifuged at 1000 xg for 10 minutes, and resuspended in 10 ml of QT-6 medium. A sample was removed for counting, the cells were then diluted further to a concentration of 1 x 10^5 cells/ml using QT-6 medium, and 65 ml of the culture was added per 225 ml flask of passaged cells.

Transfection was accomplished using cDNAs prepared as follows:

The rat GlyT-2 (rGlyT-2) clone used contains the entire sequence of rGlyT-2 cloned into pBluescript SK+(Stratagene) as an Eco RI - Hind III fragment, as described in Liu t al., J. Biol. Chem.

268, 22802-22808 (1993). GlyT-2 was then subcloned into the pRc/RSV vector as follows: A PCR fragment corresponding to nucleotides 208 to 702 of the rGlyT-2 sequence [SEQ ID NO:4] was amplified by PCR using the oligonucleotide: 5' GGGGGAAGCTTATGGATTGCAGTGCTCC 3' [SEQ ID NO:5] as the 5' primer and the oligonucleotide: 5' GGGGGGGTACCCAACACCACTGTGCTCTG 3' [SEQ ID NO:6] as the 3' primer. This created a Hind III site immediately upstream of the translation start site. This fragment, which contained a Kpn I site at the 3' end, along with a Kpn 1 - Pvu II fragment containing the remainder of the coding sequence of rGlyT-2, were cloned into pBluescript SK+ previously digested with Hind III and Sma I, in a three part ligation. A Hind III - Xba I fragment from this clone was then subcloned into the pRc/RSV vector. The resulting construct contains nucleotides 208 to 2720 of the rGlyT-2 nucleic acid [SEQ ID NO:4] in the pRc/RSV expression vector.

The human GlyT-1a (hGlyT-1a) clone used contains the sequence of hGlyT-1a [SEQ ID NO:1] from nucleotide position 183 to 2108 cloned into the pRc/CMV vector (Invitrogen, San Diego, CA) as a Hind III-Xba I fragment as described in Kim et al., Mol. Pharmacol., 45, 608-617, 1994. This cDNA encoding GlyT-1a actually contained the first 17 nucleotides (corresponding to the first 6 amino acids) of the GlyT-1a sequence from rat. To determine whether the sequence of human GlyT-1a was different in this region, the 5' region of hGlyT-1a from nucleotide 1 to 212 was obtained by rapid amplification of cDNA end using the 5' RACE system supplied by Gibco BRL (Gaithersburg, MD). The gene specific primer: 5' CCACATTGTAGTAGATGCCG 3' [SEQ ID NO:7], corresponding to nucleotides 558 to 539 of the hGlyT-1a sequence [SEQ ID NO:1], was used to prime cDNA synthesis from

human brain mRNA, and the gene specific primer: 5' GCAAACTGGCCGAAGGAGAGCTCC 3' [SEQ ID NO:8], corresponding to nucleotides 454 to 431 of the hGlyT-1a sequence [SEQ ID NO:1], was used for PCR amplification. Sequencing of this 5' region of GlyT-1a confirmed that the first 17 nucleotides of coding sequence are identical in human and rat GlyT-1a.

The human GlyT-1b (hGlyT-1b) clone used contains the sequence of hGlyT-1b [SEQ ID NO:2] from nucleotide position 213 to 2274 cloned into the pRc/CMV vector as a Hind III - Xba I fragment as described in Kim et al., *supra*.

The human GlyT-1c (hGlyT-1c) clone used contains the sequence of hGlyT-1c [SEQ ID NO:3] from nucleotide position 213 to 2336 cloned into the pRc/CMV vector (Invitrogen) as a Hind III - Xba I fragment as described in Kim et al., *supra*. The Hind III - Xba fragment of hGlyT-1c from this clone was then subcloned into the pRc/RSV vector. Transfection experiments were performed with GlyT-1c in both the pRc/RSV and pRc/CMV expression vectors.

The following four day procedure for the tranfections was used:

On day 1, QT-6 cells were plated at a density of 1 x 10^6 cells in 10 ml of complete QT-6 medium in 100 mm dishes.

On day 2, the medium was aspirated and the cells were washed with 10 ml of PBS followed by 10 ml of TBS. The TBS was aspirated, then 1 ml of the DEAE/DNA mix was added to the plate. The plate was swirled in the hood every 5 minutes. After 30 minutes, 8 ml of 80 μM chloroquine in QT-6 medium was added and the culture was incubated for 2.5 hours at 37°C and 5% CO_2. The medium was then aspirated and the cells were washed two times with complete QT-

6 medium, then 100 ml complete QT-6 medium was added and the cells were returned to the incubator.

On day 3, the cells were removed with trypsin/EDTA as described above, and plated into the wells of 96-well assay plates at approximately $2x10^5$ cells/well.

On day 4, glycine transport was assayed as described in Example 2.

EXAMPLE 2

This example illustrates a method for the measurement of glycine uptake by transfected cultured cells.

Transient GlyT-transfected cells or control ("mock") cells grown in accordance with Example 1 were washed three times with HEPES buffered saline (HBS). The mock cells were treated precisely as the GlyT-transfected cells except that the transfection procedure omitted any cDNA. The cells were incubated 10 minutes at 37°C, after which a solution was added containing 50 nM [^{3}H] glycine (17.5 Ci/mmol) and either (a) no potential competitor, (b) 10 mM nonradioactive glycine or (c) a concentration of a candidate drug. A range of concentrations of the candidate drug was used to generate data for calculating the concentration resulting in 50% of the effect (*e.g.*, the IC_{50}s, which are the concentrations of drug inhibiting glycine uptake by 50%). The cells were then incubated another 20 minutes at 37°C, after which the cells were aspirated and washed three times with ice-cold HBS. The cells were harvested, scintillant was added to the cells, the cells were shaken for 30 minutes, and the radioactivity in the cells was counted using a scintillation counter. Data were compared betwe n the cells contacted or not contacted by a candidate agent, and

between cells having GlyT-1 activity versus cells having GlyT-2 activity, depending on the assay being conducted.

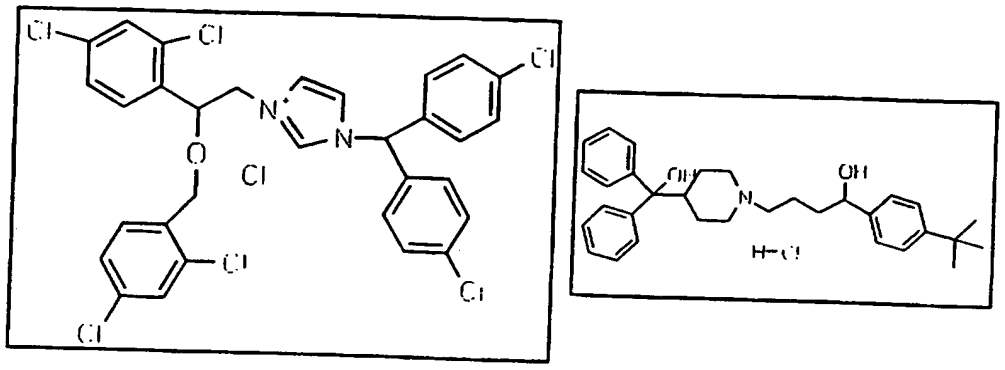
Positive control results are depicted in the bar graphs of Figures 1A and 1B, in which [^{3}H] glycine uptake is shown for mock, GlyT-1a, GlyT-1b, GlyT-1c, and GlyT-2 transformed cells. The results of the positive controls are presented as means ± SEM of a representative experiment performed in triplicate. All cell cultures transformed with any of the glycine transporters evidenced a significant increase in glycine transport activity as compared to non-transfected control cells.

EXAMPLE 3

This example illustrates the application of the method of Example 2, and the identification thereby of certain agents that regulate selectively the GlyT-1 or the GlyT-2 transporter, with respect to each other.

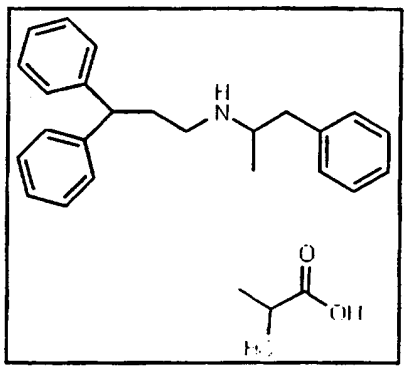
The agents recited below were tested for inhibition or enhancement of glycine transport in QT-6 cells that were transfected with pRc/CMV containing GlyT-1c [SEQ ID NO:3] or GlyT-2 [SEQ ID NO:4], and exhibited transient expression of GlyT-1c or GlyT-2, respectively, in accordance with the procedures of Examples 1 and 2 above.

ZA

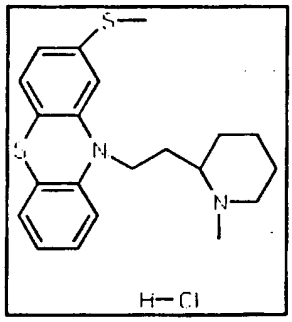


5

ZB



ZC



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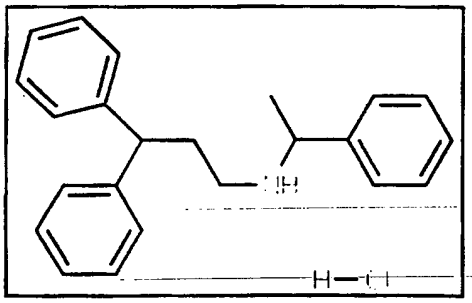
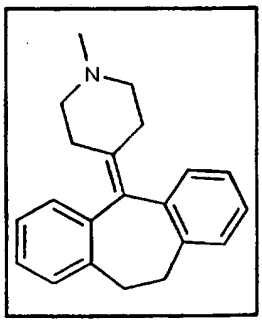


ZE



ZF

The data obtained with these compounds are as follows:

Compound	Effect Via GlyT-1c*	Effect Via GlyT-2*
ZA	pIC_{50} = 6.04	pIC_{50} = 5.51
ZB	pIC_{50} = 5.37	pIC_{50} = 4.77
ZC	pIC_{50} = 5.19	pIC_{50} = 4.85
ZD	pIC_{50} = 5.02	pIC_{50} = 4.71
ZE	pIC_{50} = 4.89	pIC_{50} = 4.68
ZF	pIC_{50} = 4.67	pIC_{50} = 4.84

* Transfected into QT-6 cells. The term "pIC_{50}" equals -log of IC_{50}, wherein IC_{50} is the concentration of drug inhibiting glycine uptake by 50%.

Accordingly, compounds ZA, ZB, ZC, ZD, and ZE are each selective for GlyT-1c relative to GlyT-2, whereas compound ZF shows the reverse selectivity.

EXAMPLE 4

This example illustrates binding assays to measure interaction of compounds with the glycine site on the NMDA receptor.

Direct binding of [^{3}H]glycine to the NMDA-glycine site was performed according to the method of Grimwood et al., Molecular Pharmacology, 41, 923-930 (1992); Yoneda et al., J. Neurochem, 62, 102-112 (1994).

Preparation of membranes for the binding test required application of a series of standard methods. Unless otherwise specified, tissues and homogenates were kept on ice and centrifugations were conducted at 4°C. Homogenizations were

conducted with an effort to minimize resulting rise in tissue/homogenate temperature. The membrane preparation included the following steps:

1. Sacrifice and decapitate four rats; remove cortices and hippocampi.
2. Homogenize tissue in twenty volumes of 0.32 M sucrose/5 mM Tris-Acetate (pH 7.4) with 20 strokes of a glass/teflon homogenizer.
3. Centrifuge tissue at 1000 x g, 10 minutes. Save supernatant. Resuspend pellet in small volume of buffer and homogenize again. Centrifuge the homogenized pellet and combine the supernatant with the previous supernatant.
4. Centrifuge the combined supernatants at 40,000 x g, for 30 minutes. Discard the supernatant.
5. Resuspend the pellet in 20 volumes of 5 mM Tris-Acetate (pH 7.4). Stir the suspension on ice for one hour. Centrifuge the suspension at 40,000 x g for 30 minutes. Discard the supernatant and freeze the pellet for at least 24 hours.
6. Resuspend the pellet from step 5 in Tris Acetate buffer (5 mM, pH 7.4) containing 0.1% saponin (w/v; Sigma Chemical Co., St. Louis) to a protein concentration of 1 mg/ml. Leave on ice for 20 minutes. Centrifuge the suspension at 40,000 x g for 30 minutes. Resuspend the pellet in saponin-free buffer and centrifuge again. Resuspend the pellet in Tris-Acetate buffer at a concentration of 10 mg/ml and freeze in aliquots.
7. On day three, remove an aliquot of membranes and thaw on ice. Dilute the suspension into 10 ml Tris-Acetate

buffer and centrifuge at 40,000 x g for 30 minutes. Repeat the wash step twice more for a total of 3 washes. Resuspend the final pellet at a concentration of 1 mg/ml in glycine-free Tris-Acetate buffer.

The binding test was performed in Eppendorf tubes containing approximately 150 μg of membrane protein and 50 nM [^{3}H]glycine in a volume of 0.5 ml. Non-specific binding was determined with 1 mM glycine. Drugs were dissolved in assay buffer (50 mM Tris-acetate, pH 7.4) or DMSO (final concentration of 0.1%). Membranes were incubated on ice for 30 minutes and bound radioligand was separated from free radioligand by filtration on Whatman GF/B glass fiber filters or by centrifugation (18,000 x g, 20 min). Filters were washed three times quickly with ice-cold 5 mM Tris-acetate buffer. Filters were dried and placed in scintillation tubes and counted. Pellets were dissolved in deoxycholate/NaOH (0.1 N) solution overnight, neutralized and radioactivity was determined by scintillation counting.

A second binding test for the NMDA-glycine site used [^{3}H]dichlorokynurenic acid (DCKA) and membranes prepared as above. See, Yoneda et al., J. Neurochem., 60, 634-645 (1993). The binding assay was performed as described for [^{3}H]glycine above except that [^{3}H]DCKA was used to label the glycine site. The final concentration of [^{3}H]DCKA was 10 nM, and the assay was performed for 10 minutes on ice.

A third binding test used for the NMDA-glycine site used indirect assessment of affinity of ligands for the site by measuring the binding of [^{3}H]MK-801 (dizocilpine; Palmer and Burns, J. Neurochem., 62, 187-196 (1994)). Preparation of membranes for the test was the same as above. The binding assay allowed s parat detection of antagonists and agonists.

The third binding test was operated to identify antagonists as follows: 100 μg of membranes were added to wells of a 96-well plate, along with glutamate (10 μM) and glycine (200 nM) and various concentrations of the ligand to be tested. The assay was started by the addition of 2.5 nM [^{3}H]MK-801 (23.9 Ci/mmol), which binds to the ion channel associated with NMDA receptors. The final volume of the assay was 200 μl. The assay was performed for 1 hour at room temperature. Bound radioactivity was separated from free by filtration, using a TOMTEC harvester. Antagonist activity was indicated by decreasing radioactivity associated with the NMDA receptor with increasing concentration of the tested ligand. Results of a positive control of this test are depicted in the graph of Figure 2A, wherein the effect of varying concentrations of the glycine-site antagonist L-689,560 (represented as the log of the molar concentration of L-689,560 on the x-axis) is shown with respect to the resultant binding of [^{3}H]MK-801, indicated in counts per minute on the y-axis. The concentration of antagonist resulting in about a 50% effect was about $5x10^{-7}$ M.

The third binding test was operated to identify agonists by performing the test as above, except that the concentration of glycine was 2 nM. Agonist activity was indicated by increasing radioactivity associated with the NMDA receptor with increasing concentration of the tested ligand. Results of a positive control of this test are depicted in the graph of Figure 2B, wherein the effect of varying concentrations of glycine (x-axis, log of the molar concentration of glycine) is shown with respect to the resultant binding of [^{3}H]MK-801 in counts per minute (y-axis). The concentration of agonist (here, glycine itself) resulting in about a 50% effect was about 10^{-6} M.

EXAMPLE 5

This example illustrates a protocol for measuring calcium flux in primary neuronal cells, which is an indication of NMDA receptor activation.

The calcium flux measurement is performed in primary neuronal cell cultures, which are prepared from rat fetal cortices dissected from pregnant rats using standard procedures and techniques that require sterile dissecting equipment, a microscope and defined medium. The protocol used was adapted from Lu et al., Proc. Nat'l. Acad. Sci. USA, 88, 6289-6292 (1991).

Defined medium is prepared in advance in accordance with the following recipe:

Components	Source (catalogue #)	Final Concentration
D-glucose	Sigma (G-7021)	0.6%
transferrin	Sigma (T-2252)	100 μg/ml
insulin	Sigma (I-5500)	25 μg/ml
progesterone	Sigma (P-6149)	20 nM
putrescine	Sigma (P-7505)	60 μM
selenium	Sigma (S-5261)	30 nM
pen-strep▲	GIBCO (15070-014)	0.5 U-0.5 μg/ml
L-glutamine*	GIBCO (25030-016)	146 mg/l
MEM°	GIBCO (11095 or 11090)	500 ml/l
F-12	GIBCO (11765)	500 ml/l

▲ pen-strep: 5,000 U/ml penicillin and 5,000 μg/ml steptomycin
* add only when MEM without L-glutamine is used
o with L-glutamine or without L-glutamine, respectively

Before starting the dissection, tissue culture plates were treated with polylysin (100 μg/ml for at least 30 minut s at 37°C) and washed with distilled water. Also, a metal tray containing two sets of sterile

crude dissecting equipment (scissors and tweezers) and several sets of finer dissecting tools was autoclaved. A pair of scissors and tweezers were placed into a sterile beaker with 70% alcohol and brought to the dissecting table. A petri dish with cold phosphate buffered saline (PBS) was placed on ice next to the place of dissection.

A pregnant rat (E15 or 16 on arrival from Hilltop Lab Animals (Scottdale, PA), E17 or 18 at dissection) was placed in a CO_2/dry ice chamber until it was unconscious. The rat was removed, pinned to a backing, the area of dissection was swabbed with 70% alcohol, and skin was cut and removed from the area of interest. A second pair of scissors was used to cut through and remove the prenatal pups in their sacs. The string of sacs was placed into the cold PBS and transported to a sterile hood.

The prenatal pups were removed from the sacs and decapitated. The skulls were then removed and the brains were carefully dislodged and placed into a clean petri dish with cold PBS. At this point, it was necessary to proceed with a dissecting microscope. The brain was turned so that the cortices were contacting the plate and the tissue between the dissector and the cortex (striatum and other brain parts) was scooped out. The hippocampus and olfactory bulb were cut away from the cortex. Then the tissue was turned over and the meninges were removed with tweezers. The remaining tissue (cortex) was placed in a small petri dish with defined media.

The tissue was chopped with a scalpel and then triturated with a glass pipet that had been fire polished. The chopped, triturated tissue was then transferred to a sterile plastic tube and continued to be triturated with a glass pipet with a finer opening. Cells were counted in a suitable counting chamber. Cells were plated at roughly 200,000 cells/well in 500 μl of defined medium in 24-well plates. To inhibit glia

growth, cultures were treated with 100 μM 5-flouro-2-deoxyuridine (FDUR, Sigma (F-0503)) or 50 μM uridine (Sigma (U-3003)) and 50 μM FDUR.

The cortical cultures for the standard calcium flux assay were grown in 24-well plates in the defined medium described above for 7 days and fed once with serum containing medium (10% heat inactivated fetal calf serum, 0.6% glucose in MEM) by exchanging half of the medium. Cultures were used after 12 days of incubation *in vitro*. The cultures were rinsed three times with HCSS (i.e. HEPES-buffered control salt solution, containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM HEPES, and 15 mM glucose, in HPLC water and adjusted to pH 7.4 by NaOH, which was also made in HPLC water). In the third wash, the culture was incubated at 37°C for 20 to 30 minutes.

Solutions containing $^{45}Ca^{++}$ (1.5×10^6 dpm/ml) and drugs for testing or controls were prepared in HCSS. Immediately before the above $^{45}Ca^{++}$ solutions were added, cultures were washed twice with HCSS, and 250 μl of $^{45}Ca^{++}$ solution per well was added, one plate at a time. The cultures were incubated for 10 minutes at room temperature, rinsed three times with HCSS, and 1 ml scintillation liquid per well was added, followed by shaking for at least 15 minutes. Retained radioactivity was counted in a scintillation counter.

Results of a standard calcium flux experiment are presented in Figure 3. Primary neuronal cortical cell cultures were incubated with $^{45}Ca^{++}$ alone (control), in the presence of NMDA (500 μM), or NMDA (500 μM) and the antagonist L689,560 (50 μM), as described above. Data presented in the bar graph of Figure 3 show the accumulation of $^{45}Ca^{++}$, and are the means ± SEM of a representative experiment (performed in triplicate) that was repeated with similar results. Accordingly, the results demonstrate that NMDA causes an increased

accumulation of $^{45}Ca^{++}$ and that this effect is block d by the glycine sit antagonist L-689,560.

EXAMPLE 6

This example sets forth a comparative study of the expression of glycine transporter genes in non-mammalian cells as compared to mammalian cells.

This comparative study included assessment of the efficiency of glycine transport in non-mammalian cells (QT-6; ATCC CRL-1078 or J.H. Steinbach, Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO) as compared to mammalian cells (CHO-K1, ATCC CCL-61; COS-7, ATCC CRL-1651; or LM, ATCC CCL-1.2), wherein a glycine transporter cDNA has been transfected into each of the aforementioned hosts using either a DEAE dextran or calcium phosphate method of transfection, as set forth hereinbelow. The glycine transporter cDNAs used were human glycine transporter 1c ("hGlyT1c"; SEQ ID NO:3) or rat glycine transporter 2 ("rGlyT2"; SEQ ID NO:4), which, prior to the transfection experiments, were inserted into vector pRc/CMV (Invitrogen) or pRc/RSV (Invitrogen), respectively, as described above in Example 1.

Media and Growth Conditions: The following three reagents were used for all cells studied in the instant comparative study:

- Hank's Balanced Salt Solution (HBSS; as set forth in Example 1), except the following chemicals were not included in this HBSS reagent: $CaCl_2$, $MgCl_2$, $MgSO_4$, and $NaHCO_3$. The HBSS reagent was purchased from GIBCO/BRL (catalog number 14180-020), which is a ten-fold concentrate. The 10x HBSS reagent was diluted to a 1x with sterile water, and subsequently stored in refrigerator.
- Trypsin/EDTA, composed of 0.05% Trypsin and 0.53 mM EDTA, purchased from GIBCO/BRL (catalog number 25300-062).
- Trypsin (0.25%), a stock solution of 2.5% trypsin was purchased from GIBCO/BRL (catalog number 15090-046), from which a 1x solution of trypsin (0.25%) was made using 1x HBSS as the diluent.

The 1x solution of trypsin was stored in 40 ml aliquots at -20°C.

Different media and growth conditions were required to grow the aforementioned cells, which different media and growth conditions are recited hereinbelow:

• Regarding QT-6 Cells: The medium used to culture QT-6 cells ("QT-6 Medium") was made fresh each week and included the following: (1) 500 ml of Medium 199 (Gibco/BRL, Catalog Number 11150-059); (2) 60 ml of tryptose phosphate (Gibco/BRL, Catalog Number 18050-021); (3) 30 ml fetal bovine serum (JRH 1210378P); (4) 6 ml penicillin/streptomycin solution(Gibco/BRL Catalog Number 15070-014); (5) 6 ml dimethylsulfoxide (DMSO; sterile) (Sigma, Catalog Number D2650).

The procedure used to grow QT-6 cells was as follows: The QT-6 cells were cultured in the above-described QT-6 medium at 5% CO_2 and at 37° in a humidified atmosphere, and subcultured twice a week at relatively high cell density (1 x 10^5 cells/ml or more), as follows: (1) each 225 cm^2 flask of QT-6 cells was washed twice with warm (37°) HBSS (5 ml each wash); (2) 2 ml of trypsin/EDTA was added to each flask and the flask was swirled; (3) the trypsin/EDTA was aspirated after 2 minutes; (4) the flask of cells was then incubated for 10 minutes at 37°, after which 10 ml of media were added and the cells were dislodged by swirling the flask and tapping the bottom of the flask; (5) the dislodged cells were then triturated, and added to a 50 ml conical tube; (6) the cells were counted with a hemacytometer, then diluted to 1 x 10^5 cells/ml; and (7) each 225 cm^2 flask was filled with 65 ml of the diluted cells.

• Regarding CHO-K1 Cells: The medium used to culture CHO-K1 cells ("CHO Medium") included the following: (1) 500 ml HAMS-F12 (Gibco/BRL Catalog Number 21016-019); (2) 60 ml FBS (JRH Catalog Number 1210378P); (3) 6 ml penicillin/streptomycin solution (Gibco/BRL Catalog Number 15070-014).

The procedure used to culture CHO-K1 cells was as follows: The CHO-K1 cells were cultured in 225 ml flasks in the above-described CHO medium at 5% CO_2 and at 37° in a humidified atmospher , and

subcultured twice a week using the following procedure: (1) Each 225 cm^2 flask of CHO-K1 cells was washed twice with warm (37°) HBSS (5 ml each wash); (2) 2 ml of 0.25% trypsin was added to each flask and the flask was swirled; (3) the trypsin was aspirated after 2 minutes; (4) each flask was then incubated 10-15 minutes; (5) each flask was monitored to determine when cells lift, after which 10 ml of fresh medium was added, the flask was tapped and the cells were dislodged; (6) the cell suspension was added to fresh flasks of media in a ratio of 1:15 (4 ml to 56 ml of CHO Medium).

• Regarding COS-7 Cells: The medium used to culture COS-7 cells ("COS Medium") included the following: (1) 500 ml DMEM (Gibco/BRL Catalog Number 11960-10); (2) 60 ml FBS (JRH Catalog Number 121037); (3) 6 ml penicillin/streptomycin solution (Gibco/BRL Catalog Number 15070-014).

The procedure used to culture COS-7 cells was as disclosed above regarding CHO-K1 cells, except that COS-7 cells are subcultured at a 1:5 ratio once per week (12 ml of suspension to 48 ml COS Medium), and are maintained at 37° in a humidified, 10% CO_2 atmosphere.

• Regarding LM Cells: The medium used to culture LM cells ("LM Medium") included the following: (1) 500 ml DMEM(Gibco/BRL Catalog Number 11960-10); (2) 60 ml FBS (JRH Catalog Number 1210378p); (3) 6 ml penicillin/streptomycin solution (Gibco/BRL Catalog Number 15070-014); (4) 6 ml L-glutamine solution (Gibco/BRL Catalog Number 25030-081).

The procedure used to culture LM cells was as disclosed above regarding CHO-K1 cells, except that LM cells are subcultured at a 1:10 ratio twice per week (6 ml of suspension to 54 ml LM Medium), and are maintained at 37° in a humidified, 10% CO_2 atmosphere.

Transfection Procedures: The cells cultured as disclosed herein above were transfected to study the properties of individual glycine transporters; accordingly, cDNA encoding each transporter was individually transfected into appropriate host cell lines, as noted above. Because it is known that a cell can have different results depending on

the transfection method used and the particular nucleic acid being transfected, to the extent practicable, each cell and transfecting DNA combination was tested with respect to two different transfection techniques, namely DEAE dextran and calcium phosphate, both of which are recited below:

• DEAE Dextran Transfection: Transfection of cells with DEAE dextran was conducted on dishes of cells, at 50 to 75% cell density, in complete medium, using the following materials and procedure:

• Solution "A":

NaCl	8.00 g
KCl	0.38 g
Na_2HPO_4	0.20 g
Tris base	3.00 g

Adjust to pH 7.5 with HCl.

Bring to 100.0 ml with distilled H_2O. Filter-sterilize; store at room temperature.

• Solution "B":

$CaCl_2 \cdot 2H_2O$	1.5 g
$MgCl_2 \cdot 6H_2O$	1.0 g

Bring to 100 ml with distilled H_2O. Filter-sterilize; store at room temperature.

• TBS+: add one ml of 100X Ca^{2+}/Mg^{2+} solution "B", 10 ml of salt solution "A"; bring to 100 ml with distilled H_2O. Filter-sterilize and store at 4°C.

• DEAE Solution: 10 mg/ml DEAE (Sigma, D-9885) in tissue culture grade H_2O; filter sterilize, store at -20°C in 10 ml aliquots.

• Chloroquine Solution: 100 mM chloroquine (Sigma, C 6628) in tissue culture grade H_2O; filter sterilize, store at -20° in 1 ml aliquots.

• Sterile DNA: DNA of interest in appropriate expression vector, 1 µg/µl.

On day 1 of a DEAE dextran transfection procedure, cells were plated for transfection the following day, as follows: 10 ml of complete media and 2 x 10^6 cells were added into a 10 cm^2 dish.

On day 2, the cells were transfected, as follows: .

1. TBS+ and complete media were warmed in water bath.
2. DEAE mixture was made, on a per dish basis: (a) 10 µg DNA was added to 1.9 ml TBS+ and mixed well; and (b) 100 µl of 10 mg/ml DEAE dextran was added and mixed well.
3. Each flask was washed twice with 10 ml of TBS+.
4. The residual TBS+ in dishes was taken out with a 10 ml pipet.
5. The DEAE mixture was added, 2 ml/dish.
6. The dishes were incubated at room temperature for 30 minutes.
7. 8 ml complete media and 10 µl 100 mM chloroquine were added.
8. The dishes were incubated at 37°C for 2-2.5 hrs.
9. The media was replaced with 10 ml complete media and returned to the incubator.

On day 3, the dishes were replated for assay, as follows:

1. The medium was removed from each dish and the cells were washed once with HBSS.
2. 1 ml trypsin/EDTA was added to each dish and the dish was tilted in all directions so the solution covered the entire bottom surface. The trypsin/EDTA was aspirated. The washed cells were allowed to stand at room temperature approximately 5 minutes. The cells were dislodged by gentle tapping of flask.
3. 5 ml complet media was added to each dish and the cells were collected.

4. The cells were counted using a hematocytometer. The cells were plated at a density of 3×10^6 cells per 96-well dish. The cells were frequently mixed while plating.

On day 4, the transfected cells were assayed as recited below.

- Calcium Phosphate Transfection: Transfection of cells with calcium phosphate was conducted on dishes of cells, at 50 to 75% cell density, in complete medium, using the following materials and procedure:

- 2x BSS (50 mM BES (Calbiochem); 280 mM NaCl; and 1.5 mM Na_2HPO_4), adjusted to pH 6.95 to 6.98 at room temperature, with 1 N NaOH; filter sterilized through 0.45 mm nitrocellulose filter (Nalgene); aliquoted and stored at -20 C.
- 2.5 M $CaCl_2$ (sterile solution, store at 4°)
- Sterile DNA: DNA of interest in appropriate expression vector, 1 µg/µl.

On day 1 of a calcium phosphate transfection procedure, cells are plated for transfection the following day, which involves combining 10 ml of complete medium and 2×10^6 cells into a 10 cm^2 dish.

On day 2, the cells were transfected, as follows:

1. The cells were re-fed with 9 ml of medium per dish approximately one hour before transfection (in case of QT-6 cells, tryptose phosphate was omitted).
2. For transfection, 10 µg of cDNA was added to 0.45 ml H_2O. 50 µl of 2.5 M $CaCl_2$ solution was added. 500 µl of 2X BSS solution was added to the mix, and vortexed immediately, and then allowed to sit at room temperature for 20 minutes (hereinafter, "DNA mix"). A fine precipitate formed.
3. The DNA mix was added to cells drop-wise and the plate was swirled, then returned to the incubator overnight. Generally, a 3% CO_2 incubator was used.

On day 3, the cells were replated for assay, as follows:

1. The cells were checked in the morning. They generally appeared slightly shrunken and precipitate was visible. The cells were re-fed with complete media and returned to routine incubation conditions.
2. After 4 hours, the cells were removed from the medium and washed once with HBSS.
3. 1 ml trypsin/EDTA was added to each dish, which was tilted in all directions so the solution covered the entire bottom surface. The trypsin/EDTA solution was aspirated. The cells were allowed to stand at room temperature approximately 5 minutes. The cells were dislodged by gentle tapping of each flask.
4. 5 ml complete medium was added in each dish and the cells were collected.
5. The cells were counted using a hematocytometer. The cells were plated at a density of about 30,000 cells per well of a 96-well plate that was previously coated with poly-lysine (Sigma). The cells were frequently mixed while plating.

On day 4, the transfected cells were assayed, as recited below.

Measurement of Glycine Transport: Measurement of uptake of [^{3}H]glycine into transfected cells generated as above, as compare to that in non-transfected cells, was undertaken using the following materials:

- HEPES buffered saline ("HBS"), pH 7.4

	final concentration
NaCl	150 mM
KCl	5 mM
$CaCl_2 \cdot 2H_2O$	1 mM
$MgCl_2 \cdot 6H_2O$	1 mM
HEPES	20 mM
NaOH	adjust to pH 7.4
Glucose (if required)	10 mM

• [^{3}H]-glycine, purchased from Amersham, Catalog Number TRK 71 (1 mCi/ml, 18.6 Ci/mmol)

• Glycine, purchased from Sigma.

Method For Measurement of Glycine Transport: On the day before a glycine uptake experiment, 3 to 6 million cells were plated per 96-well plate, i.e., at a density of about 30,000 to 60,000 cells per well, which plates were previously coated with poly-lysine (Sigma). The following steps were effected on the day of the glycine uptake experiment:

1. Cells were washed with HBS (pre-warmed HBS + glucose to 37°C).
2. 90 µl of HBS+glucose was added to wells, which were then incubated 10 minutes at 37°C.
3. 50 nM of [^{3}H]glycine with or without 10 mM unlabeled glycine was added to each well, including various concentrations of competing drug, as required.
4. The cells were then incubated 20 minutes at 37°C.
5. The wells were aspirated and washed three times with ice-cold HBS.
6. Scintillant was added, the wells were then shaken for 30 minutes and counted in Wallac MicroBeta LSC.

Analysis of Glycine Transport Data: The resulting data were defined as follows:

1. "Total transport" means the radioactivity ("cpm") associated with cells incubated in the presence of 50 nM [^{3}H]glycine in the absence of unlabeled glycine.

2. "Nonspecific transport" means the radioactivity ("cpm") associated with cells incubated in the presence of 50 nM [^{3}H]glycine and 10 mM unlabeled glycine.

3. "Specific transport" means the Total Transport less the Nonspecific transport.

The data were normalized with respect to the total protein present in an assay well, which was determined using a commercial

protein assay kit (BCA kit from Pierce (Catalog Number 23225)). Data are presented herein as specific cpm per mg protein.

The data were further manipulated to determine the "fold stimulation," i.e., the multiple needed to obtain the radioactivity associated with the glycine uptake of a transfected cell based on the results using a mock transfected cells; which manipulated data are shown in the following table. "Mock" transfected cells are prepared by transfecting an "irrelevant" cDNA (i.e., one that has no effect on glycine transport) into the host cells, rather than a glycine transporter cDNA; cDNA encoding CD8 was routinely used for this purpose.

		hGlyT1c		rGlyT2	
	Transfection Method → / Cell type ↓	DEAE	$CaP0_4$	DEAE	$CaP0_4$
Experiment 1	QT-6	30.0	3.3	33.8	26.5
	COS-7	15.3	1.1	11.7	1.2
	CHO-K1	2.1	0.0	2.2	0.0
Experiment 2	QT-6	7.7	ND	10.8	ND
	COS-7	2.6	ND	1.5	ND
	CHO-K1	1.3	ND	1.1	ND
Experiment 3	QT-6	7.5	ND	12.3	ND
	COS-7	5.2	ND	5.5	ND
	CHO-K1	2.7	ND	4.5	ND
	LM	1.5	ND	1.3	ND

As noted above, results show specific uptake, expressed as fold-stimulation compared to mock-transfected cells. The symbol "ND" signifies that the indicated experiment was not done.

In every case, the transfected mammalian cells were less efficient

than the transf cted non-mammalian cells, some comparisons of which were as profound as one-quarter to one-half the efficiency, thus showing a surprising positive property of the claimed method. For example, expression of hGlyT1c transfected using the DEAE dextran transfection method was from about 20% to about 100% more efficient when using QT-6 transfected cells as compared to COS-7 transfected cells; and from about 300% to about 1500% more efficient with respect to CHO-K1 transfected cells.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred compositions and methods may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims that follow the Sequence Listing.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Borden, Laurence A.
De Vivo, Michael
Yokoyama, Midori
Albert, Vivian R.

(ii) TITLE OF INVENTION: GLYCINE TRANSPORTER-TRANSFECTED CELLS AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Dechert Price & Rhoads
(B) STREET: P.O. Box 5218
(C) CITY: Princeton
(D) STATE: NJ
(E) COUNTRY: USA
(F) ZIP: 08543

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patenting Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US unassigned
(B) FILING DATE: 31-MAY-1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bloom, Allen
(B) REGISTRATION NUMBER: 29135
(C) REFERENCE/DOCKET NUMBER: 317743-105

(i) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 609-520-3214
(B) TELOPHASE: 609-520-3259

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2136 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCATGGTAGG AAAAGGTGCC AAAGGGATGC TGAATGGTGC TGTGCCCAGC GAGGCCACCA 240

AGAGGGACCA GAACCTCAAA CGGGGCAACT GGGGCAACCA GATCGAGTTT GTACTGACGA 300

GCGTGGGCTA TGCCGTGGGC CTGGGCAATG TCTGGCGCTT CCCATACCTC TGCTATCGCA 360

ACGGGGGAGG CGCCTTCATG TTCCCCTACT TCATCATGCT CATCTTCTGC GGGATCCCCC 420

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GGATCAGCCC CATGTTCAAA GGAGTGGGCT ATGGTATGAT GGTGGTGTCC ACCTACATCG 540

GCATCTACTA CAATGTGGTC ATCTGCATCG CCTTCTACTA CTTCTTCTCG TCCATGACGC 600

ACGTGCTGCC CTGGGCCTAC TGCAATAACC CCTGGAACAC GCATGACTGC GCCGGTGTAC 660

TGGACGCCTC CAACCTCACC AATGGCTCTC GGCCAGCCGC CTTGCCCAGC AACCTCTCCC 720

ACCTGCTCAA CCACAGCCTC CAGAGGACCA GCCCCAGCGA GGAGTACTGG AGGCTGTACG 780

TGCTGAAGCT GTCAGATGAC ATTGGGAACT TTGGGGAGGT GCGGCTGCCC CTCCTTGGCT 840

GCCTCGGTGT CTCCTGGTTG GTCGTCTTCC TCTGCCTCAT CCGAGGGGTC AAGTCTTCAG 900

GGAAAGTGGT GTACTTCACG GCCACGTTCC CCTACGTGGT GCTGACCATT CTGTTTGTCC 960

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ACAAGATCCT GGAGGCCAAG GTGTGGGGTG ATGCTGCCTC CCAGATCTTC TACTCACTGG 1080

CGTGCGCGTG GGGAGGCCTC ATCACCATGG CTTCCTACAA CAAGTTCCAC AATAACTGTT 1140

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TCCAGTACCA GCCGATCACC TACAACCACT ACCAGTACCC AGGCTGGGCC GTGGCCATTG 1800

GCTTCCTCAT GGCTCTGTCC TCCGTCCTCT GCATCCCCCT CTACGCCATG TTCCGGCTCT 1860

GCCGCACAGA CGGGGACACC CTCCTCCAGC GTTTGAAAAA TGCCACAAAG CCAAGCAGAG 1920

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CTCCTGAGGA CGGCTTCGAG GTCCAGTCAC TGCACCCGGA CAAGGCGCAG ATCCCCATTG 2040

TGGGCAGTAA TGGCTCCAGC CGCCTCCAGG ACTCCCGGAT ATAGCACAGC TGCCAGGGGA 2100

GTGCCACCCC ACCCGTGCTC CACGAGAGAC TGTGAG 2136

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2202 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCCCACACAC CCCACTCCAG CTCCGGAGCA CCCGTGCTGG GCTGCATGGG GACTGGCCGG 60

AGGGGCAGGG CCAGGGGAGC GGGTAGGCAG AGCTTCGGGA GGAGATGAGG TGAAAGTAAT 120

TGACGCTGCC CAGCCCGGCA GTGGGAGAGG CAGGGGATGC GTCAGTGTCG CGCTGGAGCT 180

GGCAGAGGTG ATGAGCGGCG GAGACACGCG GGGCTGCGAT CGCTCGCCCC AGGATGGCCG 240

CGGCTCATGG ACCTGTGGCC CCCTCTTCCC CAGAACAGAA TGGTGCTGTG CCCAGCGAGG 300

CCACCAAGAG GGACCAGAAC CTCAAACGGG GCAACTGGGG CAACCAGATC GAGTTTGTAC 360

TGACGAGCGT GGGCTATGCC GTGGGCCTGG GCAATGTCTG GCGCTTCCCA TACCTCTGCT 420

ATCGCAACGG GGGAGGCGCC TTCATGTTCC CCTACTTCAT CATGCTCATC TTCTGCGGGA 480

TCCCCCTCTT CTTCATGGAG CTCTCCTTCG GCCAGTTTGC AAGCCAGGGG TGCCTGGGGG 540

TCTGGAGGAT CAGCCCCATG TTCAAAGGAG TGGGCTATGG TATGATGGTG GTGTCCACCT 600

ACATCGGCAT CTACTACAAT GTGGTCATCT GCATCGCCTT CTACTACTTC TTCTCGTCCA 660

TGACGCACGT GCTGCCCTGG GCCTACTGCA ATAACCCCTG GAACACGCAT GACTGCGCCG 720

GTGTACTGGA CGCCTCCAAC CTCACCAATG GCTCTCGGCC AGCCGCCTTG CCCAGCAACC 780

TCTCCCACCT GCTCAACCAC AGCCTCCAGA GGACCAGCCC CAGCGAGGAG TACTGGAGGC 840

TGTACGTGCT GAAGCTGTCA GATGACATTG GGAACTTTGG GGAGGTGCGG CTGCCCCTCC 900

TTGGCTGCCT CGGTGTCTCC TGGTTGGTCG TCTTCCTCTG CCTCATCCGA GGGGTCAAGT 960

CTTCAGGGAA AGTGGTGTAC TTCACGGCCA CGTTCCCCTA CGTGGTGCTG ACCATTCTGT 1020

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TTCCCATCTC CCCGCTGTGG TCTCTGCTCT TCTTCTTCAT GCTTATCCTG CTGGGGCTGG 1440

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GGCTCTGCCG CACAGACGGG GACACCCTCC TCCAGCGTTT GAAAAATGCC ACAAAGCCAA 1980

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AGGGGAGTGC CACCCCACCC GTGCTCCACG AGAGACTGTG AG 2202

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2364 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCCACACAC CCCACTCCAG CTCCGGAGCA CCCGTGCTGG GCTGCATGGG GACTGGCCGG 60

AGGGGCAGGG CCAGGGGAGC GGGTAGGCAG AGCTTCGGGA GGAGATGAGG TGAAAGTAAT 120

TGACGCTGCC CAGCCCGGCA GTGGGAGAGG CAGGGGATGC GTCAGTGTCG CGCTGGAGCT 180

GGCAGAGGTG ATGAGCGGCG GAGACACGCG GGGCTGCGAT CGCTCGCCCC AGGATGGCCG 240

CGGCTCATGG ACCTGTGGCC CCCTCTTCCC CAGAACAGGT GACGCTTCTC CCTGTTCAGA 300

GATCCTTCTT CCTGCCACCC TTTTCTGGAG CCACTCCCTC TACTTCCCTA GCAGAGTCTG 360

TCCTCAAAGT CTGGCATGGG GCCTACAACT CTGGTCTCCT TCCCCAACTC ATGGCCCAGC 420

ACTCCCTAGC CATGGCCCAG AATGGTGCTG TGCCCAGCGA GGCCACCAAG AGGGACCAGA 480

ACCTCAAACG GGGCAACTGG GGCAACCAGA TCGAGTTTGT ACTGACGAGC GTGGGCTATG 540

CCGTGGGCCT GGGCAATGTC TGGCGCTTCC CATACCTCTG CTATCGCAAC GGGGGAGGCG 600

CCTTCATGTT CCCCTACTTC ATCATGCTCA TCTTCTGCGG GATCCCCCTC TTCTTCATGG 660

AGCTCTCCTT CGGCCAGTTT GCAAGCCAGG GGTGCCTGGG GGTCTGGAGG ATCAGCCCCA 720

TGTTCAAAGG AGTGGGCTAT GGTATGATGG TGGTGTCCAC CTACATCGGC ATCTACTACA 780

ATGTGGTCAT CTGCATCGCC TTCTACTACT TCTTCTCGTC CATGACGCAC GTGCTGCCCT 840

GGGCCTACTG CAATAACCCC TGGAACACGC ATGACTGCGC CGGTGTACTG GACGCCTCCA 900

ACCTCACCAA TGGCTCTCGG CCAGCCGCCT TGCCCAGCAA CCTCTCCCAC CTGCTCAACC 960

ACAGCCTCCA GAGGACCAGC CCCAGCGAGG AGTACTGGAG GCTGTACGTG CTGAAGCTGT 1020

CAGATGACAT TGGGAACTTT GGGGAGGTGC GGCTGCCCCT CCTTGGCTGC CTCGGTGTCT 1080

CCTGGTTGGT CGTCTTCCTC TGCCTCATCC GAGGGGTCAA GTCTTCAGGG AAAGTGGTGT 1140

ACTTCACGGC CACGTTCCCC TACGTGGTGC TGACCATTCT GTTTGTCCGC GGAGTGACCC 1200

TGGAGGGAGC CTTTGACGGC ATCATGTACT ACCTAACCCC GCAGTGGGAC AAGATCCTGG 1260

AGGCCAAGGT GTGGGGTGAT GCTGCCTCCC AGATCTTCTA CTCACTGGCG TGCGCGTGGG 1320

GAGGCCTCAT CACCATGGCT TCCTACAACA AGTTCCACAA TAACTGTTAC CGGGACAGTG 1380

TCATCATCAG CATCACCAAC TGTGCCACCA GCGTCTATGC TGGCTTCGTC ATCTTCTCCA 1440

TCCTCGGCTT CATGGCCAAT CACCTGGGCG TGGATGTGTC CCGTGTGGCA GACCACGGCC 1500

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GCCAGGCAGG CATCTATTGG CTGCTGCTGA TGGACAACTA TGCGGCCAGC TTCTCCTTGG 1800

TGGTCATCTC CTGCATCATG TGTGTGGCCA TCATGTACAT CTACGGGCAC CGGAACTACT 1860

TCCAGGACAT CCAGATGATG CTGGGATTCC CACCACCCCT CTTCTTTCAG ATCTGCTGGC 1920

GCTTCGTCTC TCCCGCCATC ATCTTCTTTA TTCTAGTTTT CACTGTGATC CAGTACCAGC 1980

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GCTTCGAGGT CCAGTCACTG CACCCGGACA AGGCGCAGAT CCCCATTGTG GGCAGTAATG 2280

GCTCCAGCCG CCTCCAGGAC TCCCGGATAT AGCACAGCTG CCAGGGGAGT GCCACCCCAC 2340

CCGTGCTCCA CGAGAGACTG TGAG 2364

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2817 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAATTCGGCA CGAGTCCGAA TCCAAAGGGG TAATGATTTA TCAAACGTGT ATTATCAGGA 60

AGATGTCAAA CGAAGGGCAC CTTGCTTCCC ACTGACGCAA ACCCGGCCTT TCCTGGGGAG 120

ATATAGAAAG CGCCTCTTGT TCCAGGGCCA AACCTAGACC AGTAGCGGGG TTTTACTCTA 180

CGGTTCAATC TGTTGTCCGC ATCAGACATG GATTGCAGTG CTCCCAAGGA AATGAATAAA 240

CCACCAACCA ACATCTTGGA GGCAACGGTG CCGGGCCACC GGGATAGCCC TCGAGCACCT 300

AGGACCAGCC CTGAGCAGGA TCTTCCTGCG GCAGCCCCCG CGGCCGCTGT CCAGCCGCCA 360

CGTGTGCCCA GGTCGGCTTC CACCGGCGCC CAAACTTTCC AGTCTGCGGA TGCGAGAGCC 420

TGTGAGGCAC AGCGGCCTGG AGTAGGGTTT TGTAAACTTA GCAGCCCCCA GGCACAAGCG 480

ACCTCTGCGG CCCTCCGGGA CTTAAGCGAA GGGCACAGCG CACAGGCCAA TCCCCCTTCC 540

GGGGCCGCTG GGGCTGGCAA CGCTTTACAC TGCAAGATTC CAGCTCTGCG TGGCCCGGAG 600

GAGGACGAGA ACGTGAGTGT GGCCAAGGGC ACGCTGGAGC ACAACAATAC CCCACCCGTG 660

GGCTGGGTGA ATATGAGCCA GAGCACAGTG GTGTTGGGTA CCGATGGAAT CGCGTCGGTG 720

CTCCCGGGCA GCGTGGCCAC CACTACCATT CCGGAGGACG AGCAAGGGGA TGAGAATAAG 780

GCCAGAGGGA ACTGGTCCAG CAAACTGGAC TTCATCCTGT CCATGGTGGG GTACGCAGTG 840

GGGCTGGGTA ATGTTTGGAG GTTTCCCTAC CTGGCCTTCC AGAACGGGGG AGGTGCTTTC 900

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CAGGGCTGTG GCATTGCGAT GCTCATCATC TCCGTCCTCA TAGCCATCTA CTACAACGTC 1080

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TCGCTGGCAA AAGGAATTAA GACATCAGGA AAAGTGGTGT ACTTCACAGC CACCTTCCCT 1500

TATGTCGTCC TGGTCATCCT CCTCATTCGA GGGGTCACCC TGCCTGGAGC TGGAGCCGGT 1560

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TCTTACAACA AATTCCATAA CAACTGCTAC AGGGACACGT TAATTGTAAC CTGCACCAAC 1740

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TCCCCACACC TCGGTTCACA GCTGTGCATG AGAGTGTTCC ATAGAAAAGT AGGACCTAAC 2760

GTAGCATGCA TTAAATCCAA CTTCCTCTCA CAAAAAAAAA AAAAAAAAAA AAAGCTT 2817

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGAAGCT TATGGATTGC AGTGCTCC 28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGGGGGTAC CCAACACCAC TGTGCTCTG 29

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCACATTGTA GTAGATGCCG 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAAACTGGC CGAAGGAGAG CTCC 24

WE CLAIM:

1. A non-mammalian cell comprising an exogenous nucleic acid encoding a glycine transporter.

2. The non-mammalian cell of claim 1, wherein the cell is selected from the group consisting of avian, fungal, insect, and reptilian.

3. The non-mammalian cell of claim 2, wherein the cell is avian.

4. The non-mammalian cell of claim 1, wherein the exogenous nucleic acid is mammalian.

5. The non-mammalian cell of claim 4, wherein the exogenous nucleic acid is human or rat.

6. The non-mammalian cell of claim 4, wherein the glycine transporter is glycine transporter-1 (GlyT-1) or glycine transporter-2 (GlyT-2).

7. The non-mammalian cell of claim 6, where the glycine transporter is human GlyT-1.

8. The non-mammalian cell of claim 7, wherein the exogenous nucleic acid is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3.

9. The non-mammalian cell of claim 6, wherein the exogenous nucleic acid is SEQ ID NO:4.

10. A method for the analysis of or screening for an agent that is an enhancer or inhibitor of glycine transport, comprising culturing separately a first and second non-mammalian cell, wherein the first and second non-mammalian cells are of the same strain and comprise an exogenous nucleic acid encoding a glycine transporter, contacting the first non-mammalian cell with the agent, and screening for the enhancement or inhibition of glycine transport into the first

non-mammalian cell as compared to glycine transport into the second non-mammalian cell that was not contacted with the agent.

11. The method of claim 10, wherein the glycine transporter is GlyT-1 or GlyT-2.

12. The method of claim 11, wherein the exogenous nucleic acid encodes GlyT-1.

13. The method of claim 12, wherein the exogenous nucleic acid comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

14. The method of claim 11, wherein the exogenous nucleic acid encodes GlyT-2.

15. The method of claim 14, wherein the exogenous nucleic acid comprises SEQ ID NO:4.

16. The method of claim 10, wherein the first or second non-mammalian cell is a QT-6 cell.

17. The method of claim 15, wherein the non-mammalian cell is a QT-6 cell.

18. The method of claim 11, wherein the drug is an enhancer or inhibitor of GlyT-1 or GlyT-2, but not of both.

19. The method of claim 11, wherein the drug is an enhancer or inhibitor of GlyT-1 and GlyT-2.

20. The method of claim 10, wherein the agent is used to treat pain, spasticity, myoclonus, muscle spasm, muscle hyperactivity, epilepsy, stroke, head trauma, neuronal cell death, multiple sclerosis, spinal cord injury, dystonia, Alzheimer's disease, multi-infarct dementia, AIDS dementia, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, attention deficit disorder, organic brain syndromes, schizophrenia, or memory or cognitive disorders.

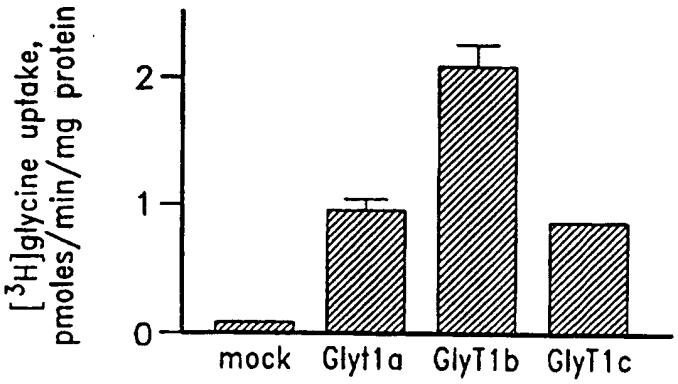

FIG. 1A

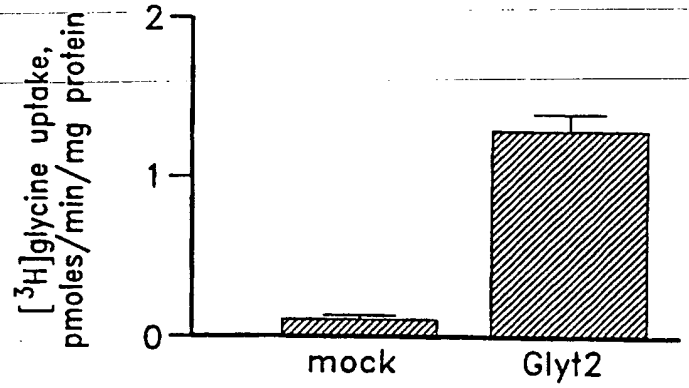

FIG. 1B

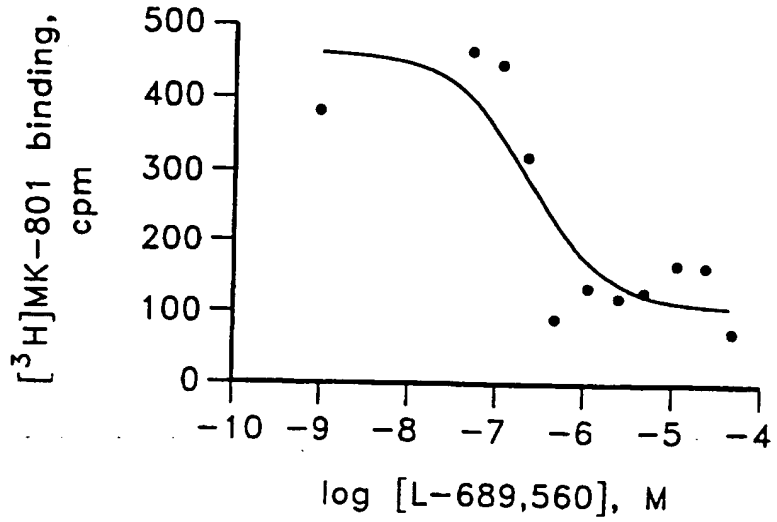

FIG. 2A

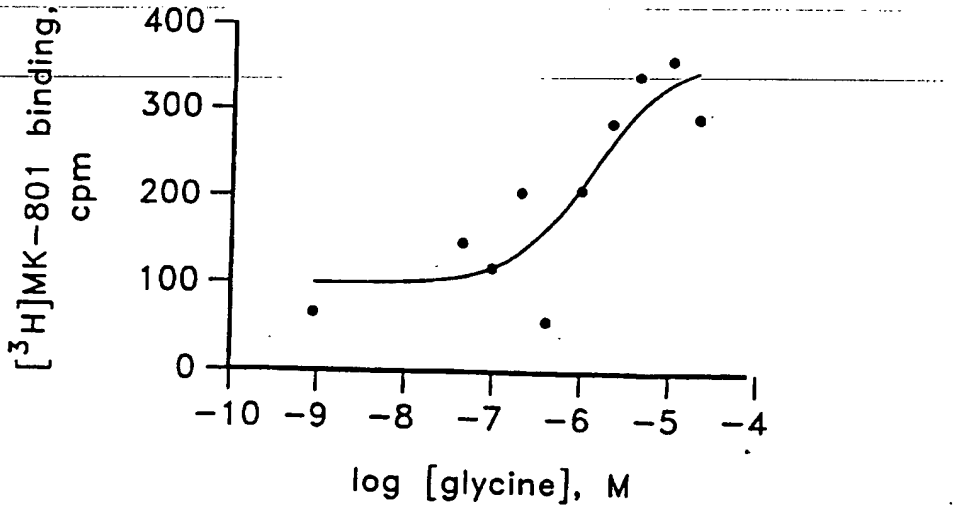

FIG. 2B

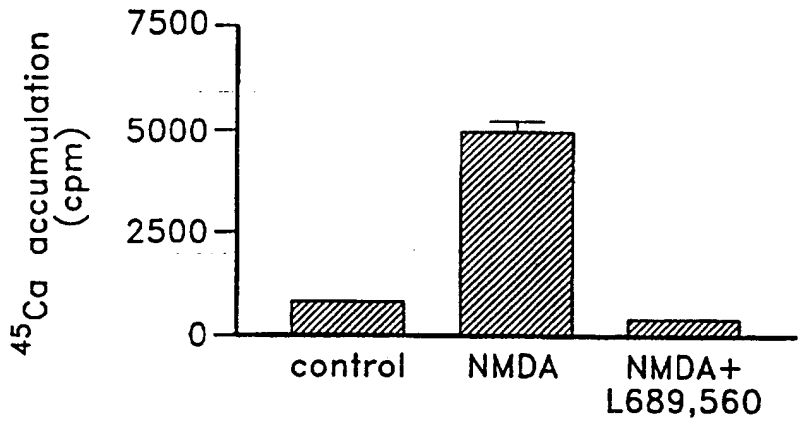
7500
5000
2500
0
^{45}Ca accumulation (cpm)
control
NMDA
NMDA+ L689,560

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09347

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 435/7.1, 69.1, 240.1, 325, 320.1, 348,349, 254.11, 29; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAPLUS, search terms: glycine transporter, glyt-1, glyt-2, SEQ ID: NOs 1-4

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	LIU et al. Characterization of the glycine transport system GLYT 1 in human placental choriocarcinoma cells (JAR). Biochim. Biophys. Acta 1994, Vol. 1194, pages 176-184, especially pages 176-182 and 184.	9, 15 ----- 10-12, 14, 16, 18-20
X ---- Y	KIM et al. Cloning of the human Glycine transporter type 1: Molecular and pharmacological characterization of novel isoform variants and chromosomal localization of the gene in the human and mouse genomes. Molec. Pharm. 1994, Vol 45, pages 608-617, especially pages 608-614.	8, 13 ----- 10-12, 14, 16, 18-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 JULY 1997	Date of mailing of the international search report 04 SEP 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KENNETH A. SORENSEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09347

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	BLAKELY et al. Expression of neurotransmitter transport from rat brain mRNA in Xenopus laevis oocytes. Proc. Natl. Acad. Science USA. December 1988, Vol. 85, pages 9846-9850, especially pages 9846-9849.	1, 10 ----- 2-7, 11-12, 14, 16, 18-20
Y	LIPSICK et al. Expression of molecular clones of v-myb in avian and mammalian cell independently of transformation. J. Virol . August 1986, Vol. 59, Number 2, pages 267-275, especially pages 268-273.	1-7, 10-12, 14, 16, 18-20
Y	SHIMADA, et al. Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. Science. 25 October 1991, Vol. pages 576-578, especially pages 576-577.	1-7, 10-12, 14, 16, 18-20.
X ---- Y	WO 93/10228 (SYNAPTIC PHARMACEUTICAL CORPORATION) 27 May 1993, pages 1-62, especially pages 47-62.	1, 4, 10, 20 ---- 2-3, 5-6, 11-12, 14, 16, 18-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09347

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 14/00, 14/47; C12N 5/00, 15/00, 15/12; G01N 33/53, C12P 21/06

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/300, 350; 435/7.1, 69.1, 240.1, 325, 320.1, 348, 349, 254.11, 29; 536/23.1, 23.5

Form PCT/ISA/210 (extra sheet)(July 1992)*